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THE COLORIMETRIC DETERMINATION OF BLOOD CALCIUM.

By JOSEPH H. ROE and BERNARD S. KAHN.

(From the Department of Chemistry, George Washington University Medical School, Washington.)

In 1926 we (1) published a colorimetric method for the estimation of blood calcium, in which the calcium is precipitated from an alkalinized trichloroacetic acid filtrate as calcium phosphate and determined as phosphate by the Benedict and Theis (2) method for the estimation of inorganic phosphorus. We have since simplified and shortened this method and in this paper we wish to report our improved procedure.

In our new procedure the calcium phosphate is precipitated, washed, dissolved in molybdic acid, and treated for color production in the same tube, thus eliminating all transfers which would require time and offer chances for error; the Fiske and Subbarow (3) method of determining inorganic phosphorus is used instead of the Benedict and Theis method, the latter procedure being retained as an optional method where a reducing agent of greater keeping qualities is desired; a more successful washing mixture for the $Ca_3(PO_4)_2$ precipitates, which eliminates one washing, has been developed; the calcium is precipitated at a higher alkalinity to remove any possibility of interference by unusual amounts of magnesium; and a number of minor changes in technique are introduced.

Procedure.

To 4 parts of 10 per cent trichloroacetic acid in an Erlenmeyer flask add 1 part of blood serum. Shake the flask until the contents are thoroughly mixed. Filter through a calcium-free filter paper (Whatman No. 42). Place 5 cc. of the filtrate in a 15 cc. conical graduated centrifuge tube, add 1 cc. of 25 per cent calcium-

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free sodium hydroxide, and let stand for 5 minutes. Add 1 cc. of 5 per cent trisodium phosphate, twirl the tube until the contents are thoroughly mixed, and set aside for 1 hour.

After 1 hour's standing centrifuge for 2 minutes. Decant the supernatant liquid and place the tube in an inverted position in a small beaker containing a mat of clean gauze or filter paper in the bottom. Allow to drain for 2 minutes, then wipe the mouth of the tube dry with a clean cloth. Add from a pipette 5 cc. of the alkaline alcoholic wash reagent, delivering the reagent in a manner that will first break up the mat of $Ca_3(PO_4)_2$ in the bottom of the tube and then wash down the sides of the tube. This is done by using a bulb pipette with a fine delivery tip. The contents of the pipette is first blown forcefully by means of the breath upon the mat of $Ca_3(PO_4)_2$ and is later directed upon the sides of the tube. If the mat of $Ca_3(PO_4)_2$ is not broken up completely by this procedure, it must be fragmented thoroughly with a clean glass stirring rod. Centrifuge for 2 minutes, then decant the wash reagent. Drain the tube as indicated above by placing in an inverted position for 2 minutes and wipe the mouth dry with a clean cloth. Add 1 cc. of the acid molybdate solution, directing the latter upon the mat of calcium phosphate in a manner that will break it up and dissolve it completely. If the mat is not broken up, tap the tube against the palm of the hand until the calcium phosphate is completely dissolved. Add from a pipette 10 cc. of distilled water, directing it into the acid molybdate with enough force to mix the contents of the tube.

In a similar graduated centrifuge tube place 10 cc. of standard phosphate solution equivalent to 0.1 mg. of calcium as $Ca_3(PO_4)_2$ and add 1 cc. of the acid molybdate solution. Now to both the standard and the unknown tubes add 0.5 cc. of aminonaphtholsulfonic acid reagent. Make each tube up to the 15 cc. mark with distilled water, mix thoroughly, and allow to stand for 10 minutes. Compare in a colorimeter in the usual manner.

If a low calcium is encountered, as indicated by a pale color being produced when the aminonaphtholsulfonic acid reagent is added, the unknown may be made up to 12 cc., instead of to 15 cc., and calculation made accordingly. If a high calcium is obtained, the solution may be decanted into a larger graduated tube and diluted to a volume that will give appropriate color comparison

with the standard. For calcium values ranging from 7 to 13 mg. per 100 cc. the regular procedure of diluting the standard and unknown to 15 cc. is entirely satisfactory. For unusually low calciums 5 cc. of standard phosphate solution (equivalent to 0.05 mg. of Ca), made up to 10 cc. with distilled water, should be taken, the amount of acid and reagent added being the same as in the regular procedure.

Calculation.

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times 10 = \text{mg. Ca per 100 cc. serum}$$

Optional Procedure with Benedict and Theis Reagent.

The calcium is precipitated and washed in the same manner as outlined above for the Fiske and Subbarow reagent. The mat of $Ca_3(PO_4)_2$, after being washed with alkaline alcohol and drained, is dissolved in 2 cc. of the acid molybdate, and 10 cc. of distilled water are added. A standard is prepared by placing 10 cc. of phosphate solution equivalent to 0.1 mg. of calcium as $Ca_3(PO_4)_2$ in a graduated tube and adding 2 cc. of the acid molybdate solution. Now to both the standard and the unknown solutions add 1 cc. of the Benedict and Theis hydroquinone-bisulfite reagent and mix the contents thoroughly. Place the tubes in a water bath at 90–95° for 10 minutes. Remove the tubes, cool by immersing in a beaker of cold water, and dilute to 15 cc. with distilled water. Mix thoroughly and compare in a colorimeter in the usual manner. The 15 cc. centrifuge tubes used cannot be boiled at 100° as Benedict and Theis do in their method for phosphorus determination, because some of the contents will boil out. Larger centrifuge tubes suitable for boiling might be used but they would be less desirable than the small 15 cc. tubes. We therefore use the small tubes and heat them in a bath at a temperature a little below the boiling point.

Reagents.

1. *Standard Phosphate Solution.* (a) *Stock Solution.*—Dissolve 2.265 gm. of pure dry monopotassium phosphate in 1 liter of phosphate-free water. 1 cc. of this reagent contains 0.5162 mg. of phosphorus equivalent to 1 mg. of calcium as $Ca_3(PO_4)_2$. Preserve with chloroform.

(b) *Phosphate Solution for Calcium Estimation.*—Pipette accurately 10 cc. of the stock phosphate solution into a liter flask and make up to the mark with phosphate-free water. 10 cc. of this solution contain 0.05162 mg. of phosphorus, equivalent to 0.1 mg. of calcium as $Ca_3(PO_4)_2$.

2. Alkaline Alcohol Wash Reagent.—In a 100 cc. graduated cylinder place 58 cc. of 95 per cent ethyl alcohol. Add 10 cc. of amyl alcohol and make up to 100 cc. with distilled water. Add 2 drops of 1 per cent phenolphthalein. Now add 5 per cent calcium-free sodium hydroxide, a drop at a time, with repeated shaking, until a distinct pink is obtained. 2 or 3 drops of the alkali are usually enough to alkalinize properly a mixture of neutral alcohols.

3. Acid Molybdate Solution.—Dissolve 12.5 gm. of ammonium molybdate, c. p., in 400 cc. of phosphate-free water. Add cautiouly 100 cc. of c. p. concentrated sulfuric acid. The final volume should be exactly 500 cc.

4. Aminonaphtholsulfonic Acid Reagent.—Dissolve 30 gm. of sodium bisulfite, c. p., and 1 gm. of sodium sulfite, c. p., in 200 cc. of phosphate-free water. Add 0.5 gm. of purified 1, 2, 4-aminonaphtholsulfonic acid[1] and stir thoroughly. A little sediment will remain in this mixture and it will be necessary to filter it. Keep in a dark bottle.

5. Hydroquinone-Bisulfite Reagent.—Dissolve 30 gm. of sodium bisulfite, c. p., and 1 gm. of highest purity hydroquinone in 200 cc. of phosphate-free water.

<div align="center">DISCUSSION.</div>

The only substance in the blood that may give interference in this method is magnesium, which, if present in unusual amounts, may be precipitated as magnesium phosphate along with the calcium phosphate and thus give an additive error. We have provided for the possible interference of unusual amounts of magnesium by alkalinizing the trichloroacetic acid filtrate to a reaction of 0.4 to 0.5 N before adding excess phosphate ions for

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[1] 1,2,4-Aminonaphtholsulfonic acid may be obtained from the Eastman Kodak Company. The directions for purifying this substance are given by Fiske and Subbarow in their article on phosphorus estimation (Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 389 (1925)).

precipitating the calcium, the magnesium being removed as $Mg(OH)_2$ by this excess of hydroxyl ions. With this procedure no interference was experienced when magnesium was added to a pure calcium solution in amounts equivalent to 30 mg. per 100 cc., which is 15 to 20 times the amount found in normal blood. The possibility of interference by unusual amounts of magnesium is not provided for in the procedure of Kuttner and Cohen (4) who modified our original procedure to obtain a micro method for blood calcium. These authors use a mixed alkali-phosphate reagent for precipitating calcium, which will give correct values with bloods containing normal amounts of magnesium, but does not provide for possible increased amounts of magnesium. To be sure no interference from magnesium occurs, it is necessary to alkalinize first before adding the precipitating phosphate ions.

In determining a suitable solution for washing the calcium phosphate precipitates we experienced considerable difficulty. The requirement for this purpose is a solution in which calcium phosphate is highly insoluble and sodium phosphate, the precipitant, is readily soluble. Solutions of ammonium hydroxide, sodium hydroxide, saturated calcium phosphate, and varying concentrations of alkaline alcohols and acetone, were tried. Of these substances a mixture of 55 parts of ethyl alcohol, 10 parts of amyl alcohol, and 35 parts of water, made just alkaline to phenolphthalein, proved to be the most satisfactory. The amyl alcohol was introduced to lower the surface tension of the mixture; this brings about a very successful washing of the calcium phosphate particles and an excellent drainage of the centrifuge tubes after decanting the wash liquid. With this mixture a second washing, as in our original procedure, is unnecessary, one washing giving excellent results.

As a reducing reagent for phosphomolybdic acid we prefer the aminonaphtholsulfonic acid reagent of Fiske and Subbarow (3). This is a rapid reducing reagent, producing a satisfactory color at ordinary temperatures, and is specific for phosphomolybdic acid. In laboratories where only an occasional blood calcium determination is made, the hydroquinone-bisulfite reagent of Benedict and Theis may be more desirable because of its superior keeping qualities. The latter reagent also has a slight advantage in that it produces a deeper blue color. For these reasons we have out-

lined the use of Benedict and Theis' reagent as an alternative procedure, but we do not recommend this reagent where a volume of work is done, as it involves the additional step of boiling to produce maximum color. The aminonaphtholsulfonic acid reagent will keep for 2 weeks according to Fiske and Subbarow. For the practically pure phosphate solutions used in this method the Fiske and Subbarow reagent will give satisfactory results for much longer than 2 weeks. The undesirable result of ageing of this reagent is that it develops a yellowish tinge and imparts a shade of green to the colors produced by it when old. This is not a serious objection since the standard and unknown solutions are treated identically. We have obtained accurate results with a reagent that was kept in a dark bottle for 1 year. It is desirable however, to have this reagent freshly prepared every 2 or 3 months, since the shade of blue obtained is better with a freshly prepared reagent.

We have found the stannous chloride reagent recommended by Kuttner and Cohen (4) undesirable because it reduces molybdic acid as well as phosphomolybdic acid. In using this reagent, which lacks specificity for phosphomolybdic acid, accurate results cannot be obtained unless the amount of the reagent introduced into the standard and unknown solutions is very accurately measured. As indicated in Kuttner and Cohen's paper the non-changing zone of color production with their stannous chloride reagent is between 0.02 and 0.022 per cent. This is an exceedingly narrow zone in which to work. In our hands variations of 0.1 cc. in the addition of this reagent gave errors in excess of 10 per cent.

The most favorable hydrogen ion concentration for color production with the aminonaphtholsulfonic acid reagent is between 0.4 and 0.9 N; with the hydroquinone-bisulfite reagent between 0.9 and 1.9 N. In these zones of acidity there is no variation in the color intensities obtained with the reagents as indicated. In our revised procedure the calcium phosphate precipitates are dissolved in molybdic acid of a concentration that will bring the reaction of the unknown solutions about to the mid point of the zone characteristic of the reagent used. Dissolving the calcium phosphate precipitates in a more dilute molybdic acid solution was tried, as it would eliminate the one step of adding water after

adding the stronger molybdic acid solution, but the use of two molybdate solutions was found unsatisfactory because the more dilute molybdate solution does not keep well. We use one molybdic acid solution of a concentration of approximately 7 N, which will keep for an indefinite time. The use of one molybdate solution also has an additional advantage in that, if it should become contaminated with impurities, no great error would result, since the same amount of the same solution is added to both the standard and the unknown.

As shown in our original paper (1) the solubility of calcium phosphate increases above pH 13. To depress this solubility and bring about maximum precipitation we use a concentration of 0.02 M sodium phosphate (produced by adding 1 cc. of 5 per cent $Na_3PO_4 \cdot 12H_2O$). This concentration of sodium phosphate brings about maximum precipitation in alkalinities up to 2 N; half this concentration of phosphate ions gives maximum precipitation up to 0.7 N. In our revised procedure precipitation is carried out at around 0.4 N alkalinity, hence there is a very liberal margin of safety in securing maximum precipitation of calcium phosphate in this method.

The sodium phosphate and sodium hydroxide solutions used must be free from calcium and silicates, either of which will give a plus error. The production of silicates is unavoidable in ordinary glass bottles containing alkaline solutions; but this difficulty may be overcome by occasionally filtering the sodium hydroxide and sodium phosphate solutions through calcium-free filter paper, or by being careful not to agitate the contents of the bottles before using, and pipetting only from the clear supernatant layers that are free from silicates.

This revised procedure is simple and rapid, and we believe it to be more accurate than any other method for the estimation of blood calcium. It is not dependent upon a balancing of compensating errors as is found in other methods; and we would especially emphasize the fact that colorimetry is the logical procedure for estimating blood calcium since calcium is present in the blood in relatively small amounts. We have found this procedure adaptable to the determination of very small amounts (0.02 mg.) of calcium. We are applying it to the development of a micro method, the possibilities of which we expect to publish later.

Blood Calcium Determination

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THE CONCENTRATION OF ACID AND BASE IN THE SERUM IN NORMAL PREGNANCY.

By HARRY C. OARD and JOHN P. PETERS.

(From the Department of Internal Medicine of Yale University and the Medical Service of the New Haven Hospital, New Haven.)

INTRODUCTION.

In 1912 Hasselbalch and Lundsgaard (28) found a lowering of the alveolar carbon dioxide tension in the later stages of pregnancy. This finding was quickly confirmed by others, notably Leimdorfer, Novak, and Porges (35) and Hasselbalch and Gammeltoft (27). That there is a reduction of the alkaline reserve in pregnancy as evinced by lowered alveolar carbon dioxide tension and lowered plasma or serum carbon dioxide tension, content, and combining power has been established by a large number of observers (12, 27, 28, 30, 36, 37, 39, 51, 58, 68). This change is demonstrable as early as the 2nd month of pregnancy (27), and Cook (11) detected a lowered plasma carbon dioxide-combining power as early as 3 days following the first missed menstrual period.

A reduction of the blood alkaline reserve may be the result of one or the other or of a combination of both of two factors: namely, an unusual collection of acid in the blood, or a reduction of the fixed base of the blood.

Along with the lowered alveolar carbon dioxide tension, Hasselbalch and Gammeltoft (27) found an increased ammonia nitrogen to total nitrogen ratio in the urine. They considered that these reactions served to maintain the pH of the blood constant in spite of increased acid production in the pregnant organism. This belief that there is in pregnancy an increase of acid in the blood has been embodied in the term "acidosis of pregnancy." The increased ammonia coefficient and the ease of production of ketonuria in pregnancy have frequently been advanced in support of that theory (49, 50, 56). Bokelmann and Bock (7) believe they have proved this by a study in which they found that the blood acetone of pregnant individuals on a high fat diet increased 63 per cent while that of normal individuals increased only 10 per cent. Bokelmann (6) has also found an increase of 2.7 mg. per cent of blood lactic acid in pregnancy. Stander and Radelet (59), on the other hand, have found no change in blood lactic acid or uric acid in normal pregnancy. Because on a given carbohydrate-free diet, pregnant individuals developed ketonuria, which could be checked by additional carbohydrate, while non-pregnant women on the

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same diet did not develop ketonuria, Kleesatel (31) concludes that in pregnancy an unusually large quantity of carbohydrate is required for the combustion of a given amount of fat.   He ascribes this to a change in the secretory function of the liver cells.

On the other hand, Harding and Allin (26) have pointed out that the mere production of ketonuria by high fat or by carbohydrate-poor diets is not satisfactory evidence of a lowered ketogenic threshold, especially if careful quantitative checks are not made.   They found not only no decrease of the "threshold of ketonuria" in pregnancy, but also that, "Diets which theoretically should produce a large excretion of acetone may only produce a ketonuria slightly above the threshold value."   They find no evidence that the lowered alkaline reserve of pregnancy is the result of unusual ketogenesis.

Against the theory of increased acid, Marrack and Boone (39) in 1923 wrote as follows: "If appreciable amounts of abnormal acid were present in the plasma of pregnant women this should reveal itself by an excess of kations; this we have not found. . . .   Such lowering of the dissociation curve as we have found appears to be due to deficiency of alkali rather than accumulation of abnormal acids in the blood."

It is apparent from the foregoing that no adequate solution of the problem is yet available.   The chief difficulty lies in the fact that all approaches have been by more or less indirect methods. Peters, Bulger, Eisenman, and Lee (43) have described a more direct method for determining the total acid-base equilibrium of serum.   The results obtained by the application of that method to the serum of normal pregnant individuals are here presented.

Methods Employed.

From the material in the dispensary and in the wards of the New Haven Hospital gravid patients were selected who throughout their pregnancy showed no signs of abnormality.   The criteria were: absence of obvious disease conditions, blood pressure below 130 systolic, negative urine (except for some cases exhibiting a minimal albuminuria without other signs), normal eye grounds, absence of edema, absence of vomiting except for the usual morning sickness, and uncomplicated delivery and convalescence.   All patients received ordinary mixed diets.   Blood was drawn anaerobically and without stasis from an arm vein, usually 3 or 4 hours after breakfast.

The techniques used in handling the blood have been described in detail elsewhere (46).

One fraction was treated with a few crystals of sodium oxalate. This whole blood was used for the non-protein nitrogen which was determined by subjecting a protein-free filtrate obtained by trichloroacetic acid precipitation to micro digestion by the usual procedure. The ammonia was distilled into 0.02 N acid and was estimated by titration with 0.02 N alkali.

A second fraction of the blood was defibrinated without contact with air by the method of Eisenman (15). Cell volumes were determined in duplicate on the defibrinated blood with a Daland hematocrit of the type manufactured by the International Equipment Company for their centrifuge. This was rotated at about 2000 R.P.M. until the cells were homogeneous and translucent and showed no further tendency to reduction of volume. Duplicate estimations usually differ by considerably less than 1 volume per cent, and it is probably justifiable to assume that for comparative purposes this represents the upper limit of error in the actual determination.

This serum of the defibrinated blood was then separated from the cells with precaution against contact with air (2).[1]

Serum carbon dioxide content was determined by the method of Van Slyke and Neill (65) in a carefully calibrated, water-jacketed, Van Slyke constant volume pipette.

A third fraction of the blood was allowed to clot without contact with air in a centrifuge tube, and the serum was separated by the same technique employed with the second fraction. This serum and the remainder of that from the defibrinated blood were then used for chloride, inorganic phosphorus, serum protein, and total base determinations.

Chlorides were determined by the latest procedure of Van Slyke (63); inorganic phosphorus by the method of Benedict and

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[1] The technique for stoppering the centrifuge tubes used in this operation has been improved in the following manner: a No-Air rubber stopper of the type used for vaccine bottles, through which a No. 26 hypodermic needle has been introduced, is inserted into the tube so that the excess of oil at the top is forced out through the needle. The cap part of the stopper is then turned down and the needle is withdrawn. In this way the tube is perfectly sealed and the danger of the cork flying out during centrifugation is obviated. To prevent exposure of the serum after centrifugation, oil is injected with a fine hypodermic needle through the stopper as the stopper is withdrawn.

Theis (5); total base by a modification (46) of Stadie and Ross' (57) adaptation of the Fiske (19) urine method.

For the determination of serum proteins about 0.5 cc. of serum was subjected to the macro-Kjeldahl procedure with an extra digestion for $\frac{1}{2}$ hour following the addition of 0.5 cc. of superoxol. From the total nitrogen the non-protein nitrogen was subtracted. The remainder was multiplied by the usual factor, 6.25. Since the serum non-protein nitrogen is ordinarily about three-fourths that of the whole blood, the value of the serum proteins is about 0.05 per cent low when the whole blood non-protein nitrogen is subtracted from the total serum nitrogen.

Calculations Employed.

Volumes per cent of CO_2 were converted into milli-equivalents present as bicarbonate in 1000 cc. of serum by the following equation:

$$B \text{ (as } BHCO_3) = 0.4225 \ CO_2$$

This was derived from the Henderson-Hasselbalch equation as follows:

$$(1) \qquad pH = pK_1 + \log \frac{BHCO_3}{H_2CO_3}$$

where

(2) $BHCO_3 = CO_2 - H_2CO_3$; $CO_2 =$ volumes per cent CO_2; and H_2CO_3 = dissolved CO_2.

Therefore

$$pH = pK_1 + \log \left(\frac{CO_2 - H_2CO_3}{H_2CO_3} \right)$$

$$(3) \ \text{Or } H_2CO_3 = \frac{CO_2}{\text{antilog } (pH - pK_1) + 1}$$

From (2) and (3)

$$(4) \qquad BHCO_3 = CO_2 - \frac{CO_2}{\text{antilog } (pH - pK_1) + 1}$$

Assuming pH = 7.35 and pK_1 = 6.10

$$(5) \qquad B \text{ (as } BHCO_3) = \frac{CO_2 - \dfrac{CO_2}{18.78}}{22.4} \times 10$$

where 22.4 = gas constant.

Solving (5) we find

$$B \text{ (as } BHCO_3) = CO_2 \times 0.4225$$

Serum proteins were converted into milli-equivalents per liter by the following equation (64):

$$BP = 1.072 \times gm. \text{ protein per cent} \times (pH - 5\ 04)$$

This becomes BP = 2.476 × gm. protein per cent, when pH = 7.35.

Inorganic phosphorus in mg. per 100 cc. was reduced to milli-equivalents per liter by the factor, $\dfrac{18}{31.04}$, according to L. J. Henderson's (29) estimate of the proportions of primary and secondary phosphate in the blood.

Total acid is calculated as the sum:

$$TA = BP + BHCO_3 + BCl + BPO_4$$

where BP, $BHCO_3$, BCl, and BPO_4 represent milli-equivalents of base combined with protein, CO_2, chlorides, and inorganic phosphorus respectively, and TA, therefore, the total base-combining power of the inorganic acids and of the protein of the serum.

The difference between this and the total base, which in the tables has been termed "undetermined acid," represents the amount of base present as sulfates and the salts of organic acids. Failure to determine the sulfur makes the total sum of the acids incomplete, and to that extent any conclusions concerning the acid-base balance must be considered tentative. Under ordinary circumstances the concentration of sulfur is negligible. Denis and King (13) have shown that the blood sulfate is not altered in pregnancy.

Results.

Tables I and II present the results. In Case 2933 there was present a very early decompensation on the basis of an old rheu-

TABLE I.

Acid-Base Equilibrium in Normal Pregnancy.

Case No.	Age (yrs.)	Para.	Wks. gravid.	Blood pressure.	Urine Albumin	Urine Casts	Urine Sugar	Blood non-protein N. (mg. per 100 cc.)	Serum proteins (per cent)	BP (mM*)	BHCO₃ (mM)	BCl (mM)	BPO₄ (mM)	Total acid. (mM)	Total base. (mM)	Undetermined acid. (mM)	Cell volume. (per cent)	Remarks.
61528	41	1	38	130/78	+	0	0	32	5.70	14.0	21.0	101.8	2.9	140.5	146.5	6.0	37.0	
61492	23	1	39	110/65	0	0	0	20	5.81	14.7	22.7	100.8	2.7	140.9	140.5	1.6	33.0	
65672	22	1	28	120/78	+	0	0	25	6.17	15.3	22.9	103.2	2.0	143.4	147.4	4.0		
31392	24	5	18	110/80	+	0	0	12	5.93	14.7	22.8	103.9	2.2	143.5	145.6	2.1		
2933	20	2	40	150/68	0	0	0	27	6.09	15.7	20.5	105.8	2.5	143.9	145.9	2.0	38.9	Multiple pregnancy.
49430	35	8	32	120/75	0	0	0	23	5.97	14.8	21.9	103.3	1.9	141.9	149.3	7.4	34.6	
67898	25	3	27	110/60	0	0	0	21	6.14	15.2	22.7	103.2	1.6	142.7	147.8	4.1	31.7	
67867	25	3	24	110/50	0	0	0	14	6.95	17.2	20.6	104.8	1.9	144.5	146.2	1.7	32.8	
67924	24	1	16	105/70	0	0	0	23	6.82	16.9	22.2	104.2	1.7	145.1	145.5	0.4	34.2	
66517	26	1	40	120/60	+	0	0	28	5.80	16.4	23.4	103.3	2.7	143.8	145.0	1.2	33.5	2 days ante partum.
66436	26	1	0	115/65	0	0	0	33	6.84	16.9	21.8	102.8	2.3	147.0	147.8	0.1	34.0	9 " post
66436	23	1	37	130/70	0	0	0	21	5.72	14.2	21.2	105.3	2.8	144.1	144.6	0.5	34.1	25 " ante
67359	23	1	0	130/70	0	0	0	25	6.73	16.6	27.6	100.4	2.5	147.1	156.0	8.9		18 " post
67359	23	2	31	120/70	0	0	0	26	6.59	16.5	22.5	105.4	2.3	146.4	143.7	2.3	34.9	9 wks. ante
67359	23	2	0	125/70	0	0	0	32	7.36	18.2	24.7	104.6	1.9	149.4	150.0	0.6	34.9	11 days post
61523	16	0	0	120/65	0	0	0	23	7.22	17.8	27.0	100.2	2.4	147.4	152.0	5.0	38.2	Hemorrhoidectomy.
Maximum								32	6.95	17.2	23.4	105.8	2.9	149.4	149.3	7.4	38.9	
Minimum								14	5.70	14.0	20.6	100.8	1.6	140.5	142.5	0.4	31.7	
Average								23	6.18	15.2	22.1	103.7	2.3	143.4	146.1	2.8	34.5	

* mM = milli-equivalents per liter

matic heart lesion, but this was successfully relieved by digitalization and the patient had an uncomplicated delivery of normal twins 48 hours after the blood specimen was taken. Her convalescence was uneventful. Two studies, one before and one after delivery, were obtained on each of three individuals (Cases 66517, 66436, and 67359).

Determinations of total base were made on ten normal adults, all of whom were engaged in their ordinary pursuits and were free from known disease process (Table II).

TABLE II.

Total Base Concentration in Normal Sera.

Case No.	Subject.	Date.	Sex.	Age.	Total base
		1927		*yrs.*	*mM**
1	D.M.	Nov. 15	Male.	29	154.4
	"	" 21	"	29	157.2
	"	" 28	"	29	155.7
2	H.C.O.	" 10	"	27	153.0
3	F.S.B.	" 12	"	30	153.6
4	L.D.	" 18	"	27	152.5
5	M W.	" 20	"	30	155.0
6	E.D.	" 20	"	26	153.0
7	A.J.E.	" 10	Female.	28	153.0
8	R.S.I.	Dec. 2	"	31	152.5
9	H.D.	Nov. 13	"	15	158.0
10	A.A.	Sept. 2	"	16	152.0
Maximum.					158.0
Minimum					152.5
Average					153.8

* mM = milli-equivalents per liter.

One complete study was made on serum from a young, non-pregnant female 10 days convalescent from an operation for hemorrhoids. Her hemoglobin and blood cell counts were normal.

DISCUSSION.

Analysis of Results.—Table III presents the values obtained for the various ions in both pregnant and non-pregnant individuals by the authors and by others who have published studies on elec-

TABLE III.

Serum Electrolytes in Pregnant and Non-Pregnant Normals.

No. of cases. Pregnant.	Serum proteins, gm. per cent.			BHCO₃, mм.			BCl, mм.			BPO₄, mм.			Total base, mм.			Observer.
	Max.	Min.	Aver.	Max.	Min.	Aver.	Max.	Min.	Aver.	Max.	Min.	Aver.	Max.	Min.	Aver.	
12	6.95	5.70	6 18	23.4	20 6	22.1	105.8	100.8	103.7*	2 9	1 6	2.3†	149 3	142 5	146 1	Oard (present study).
17							106.7	89 7	99.9‡	2 2	0 6	1.5§	157.8	137.1	145 9‖	Krebs and Briggs (33).
17				27.5	24 0	20.5	106.0	95.0	100.9‡				147	141.4	144 5‡	Marrack and Boone (39).
16				25.4	17.4	21.8	103.0	92 4	96 9‡	2.6	1.6	1.8§	169 3	151.3	159.5	Denis and King (13).
5										1.7	1.3	1.6§				Stander, Duncan, Sisson (58).
32	7.78	5.70	6.50							2.3	1.3	1.7§				de Wesselow (66).
88	7.82	6.06	7.02**													Plass and Bogert (47).
84																" " Matthew (48).
6	6.92	5.85	6.32													Zangemeister (69).
6	6.88	5.99	6.49													Dienst (14).
18	7.06	6 00	6.54													Landsberg (34).

Non-pregnant.															Reference
15	7.77	5.78	6.59††	30.9	21.8	25.8	110.1	100.0	103.5*	2.7	1.7	2.3†	161.0	147.0	155.7	Peters, Bulger, Eisenman, Lee (43).
10															158.0	Kramer and Tisdall (32).
12													158.0	152.5	153.8	Present study.
2††						106.7		95.8	102.5‡				158.3	146.6	153.6	Briggs (8).
5										1.8	1.4	1.5§				Stander, Duncan, Sisson (58).
91	7.83	6.45	7.04							2.0	1.5					Tolstoi (62).
17	7.78	6.96	7.42							2.3	1.3	1.7§				de Wesselow (66).
15	8.39	6.63	7.45													Plass and Bogert (47).
10																" " Matthew (48).
9	7.91	6.94	7.17										158.5	147.4	153.5	Peters et al. (46).
8	7.25	6.78	7.01													Salvesen and Linder (54).
2	8.36	7.94														Dienst (14).
																Landsberg (34).
																Zangemeister (69).
1		7.22		27.0					100.2			2.4†			152.0	Oard (present study).

* Van Slyke method.   †Benedict-Theis method.   ‡Whitehorn (67) method.   §Bell-Doisy method.   ‖Eight cases.   ¶Na in mμ + 12.4 mμ. See text.   **Average of cases from 3 to 6 months gravid.   ††Probably incomplete digestion.   See text.   ‡‡Selected as probable normals.

trolytes concerned in the present work. Results by different workers using similar methods are in close agreement. With one exception, discrepancies can be explained by the differences in techniques employed; the apparent differences in BPO_4 are explained by the fact that the Bell-Doisy (4) method gives lower values than the Benedict-Theis (5). By the addition of superoxol, it has been found in this laboratory that more complete digestion is effected in the Kjeldahl procedure for serum proteins. Failure to obtain complete digestion probably accounts for the lower values obtained by most workers as compared to those obtained by Plass and Matthew (48), and recent determinations from this laboratory (46). The literature contains no data for direct total base determinations in pregnancy. Where the values listed have not been obtained from direct determinations of sodium, calcium, magnesium, and potassium (as was the case with Krebs and Briggs (33) and Denis and King (13)) but from determinations of sodium alone (Marrack and Boone (39)), the value for total base has been calculated by adding 12.4 mM to the value for sodium. That this is legitimate has been proved by Briggs (8), Krebs and Briggs (33), and Denis and King (13), who have shown that the fraction of total base present as calcium, magnesium, and potassium varies insignificantly in health, in disease, or in normal or abnormal pregnancy.

It is evident from Table III that the values for the various ions in pregnancy found in the authors' studies are closely in agreement with those of many other workers. The one exception, total base by Denis and King (13), will be discussed later. A large number of independent observers are likewise in close agreement as to the range of fluctuation and the average values of the individual ions in non-pregnant normals. Table III, therefore, affords a basis for comparing the various serum electrolytes in the pregnant with those in the non-pregnant condition.

The reduction of serum proteins in pregnancy first observed by Zangemeister (69) in 1903 has been abundantly confirmed by others (14, 34, 47, 48) and by the authors. Plass and Bogert (47) and Plass and Matthew (48) have recently found that there is a progressive decrease of serum protein from the 3rd month of pregnancy to the 6th, after which there is a gradual rise, which

does not, however, restore the normal level until several weeks post partum.

A consistent decrease of about 4 mM (10 volumes per cent) $BHCO_3$ in pregnancy has been found by the authors, a finding
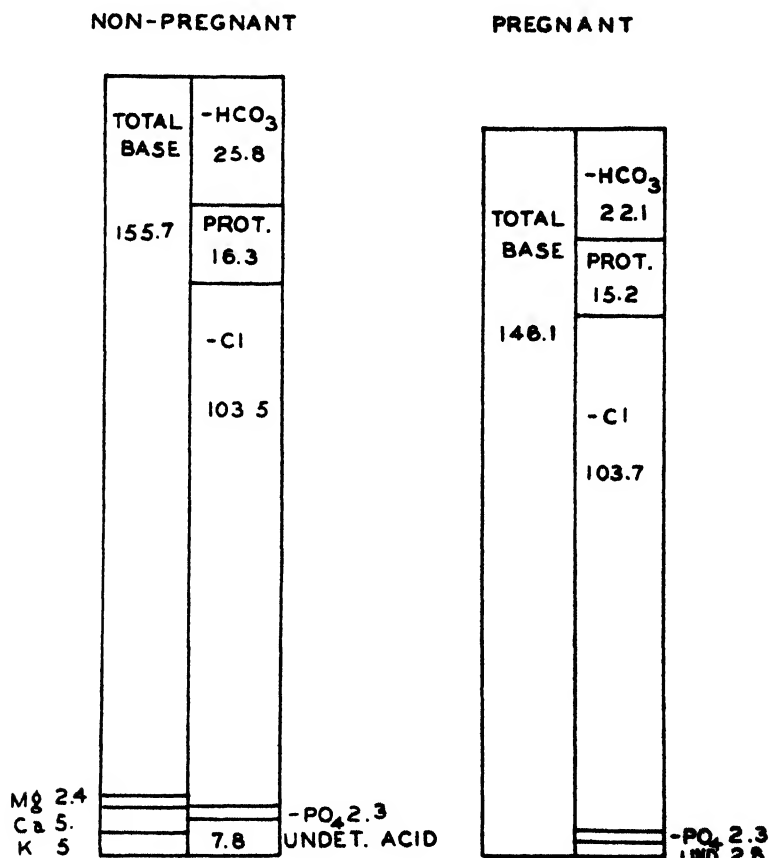

Fig. 1.

which substantiates Hasselbalch and Gammeltoft (27), Denis and King (13), and Marrack and Boone (39).

No appreciable changes of chlorides or inorganic phosphate have been found in the authors' series. This confirms the findings

of Krebs and Briggs (33), Marrack and Boone (39), and Denis and King (13).

Total base in pregnancy is lowered about 5 per cent (8 mM) from the normal, the average value in pregnancy being about 146 mM and that for the normal 154 mM (Fig. 1 and Table III). Furthermore, in pregnancy the maximum value, 149 mM, found by the authors, exceeds by only 2 mM the minimum value, 147 mM, observed by Peters, Bulger, Eisenman, and Lee (43) in normals. The findings of Denis and King (13) do not agree with the above. Their values for pregnancy would be high even for normals. Without more definite knowledge of their technique in handling blood, it can only be pointed out that they are at variance with three other groups of workers, Krebs and Briggs (33), Marrack and Boone (39), and the authors.

Pathogenesis of Acid-Base Changes.

Fetal Requirements.—The work here presented has thrown no light on the cause of the reduction of fixed base in the serum in pregnancy. Shohl (55) has calculated that the fetus requires, during the last 100 days of gestation, a daily excess of 8.5 milliequivalents of basic radicles over acid. This he believes accounts for the "acidosis of pregnancy." Harding (25) does not concur in this. He points out that the reduction of reserve alkali has repeatedly been demonstrated in the 3rd month (11, 12, 27, 28). In Case 31392 (Table I) the reduction of fixed base was fully developed by the 18th week.

Acid Production and Excretion.—Marrack and Boone (39) found in the serum of pregnancy an excess of 18 mM of anion unaccounted for by inorganic phosphorus, chloride, and bicarbonate. They assumed that this excess consisted chiefly of protein as anion. From the work of Plass and Bogert (47), Plass and Matthew (48), and the author, we may assign a value of about 15 mM to the anion present as serum protein. This leaves an excess, on the basis of Marrack and Boone's findings, of about 3 mM of undetermined acid. The average value found in the authors' work for undetermined acid is also 3 mM. The undetermined acid in pregnancy is less than half the amount found by Peters, Bulger, Eisenman, and Lee (43) for normals (Table III and Fig. 1).

Harding and Allin (26), as a result of careful metabolism studies, conclude that the normal mechanism of ketogenesis is not disturbed in pregnancy. They point out that those observers who claim to have demonstrated a greater tendency to ketone production have failed to take into account the raised metabolism of pregnancy, especially during the later stages, and the amount of antiketogenic material removed from the metabolic mixture by the fetus for the formation of new tissue. These two factors may cause the appearance of ketosis on diets which would not have the same effect in a normal individual. Even if we were to grant such a mild ketosis as is reputed to exist in pregnancy, one would not expect a reduction of base to ensue. In studies of acid-base equilibrium in diabetes, Peters, Bulger, Eisenman, and Lee (45) found that in moderate or severe, slowly or rapidly developing ketoses, base was released first by bicarbonate and next by chloride to neutralize ketone acids. Except in an overwhelming acidosis, the normal concentration of base in the serum remained unaffected. The same phenomena have been demonstrated by Gamble, Ross, and Tisdall (23) in fasting. These two groups have, however, conclusively shown that the depletion of body stores of base by acid excretion results in diuresis sufficient to maintain the concentration of serum base approximately at the normal level. This close relationship between total base concentration and water metabolism is also substantiated by many others. Cohnheim (10), for example, found that weight lost by excessive sweating during exercise could not be restored by drinking distilled water. Only when salt so lost was replaced, could weight be recovered. The diuresis produced by acidifying salts was found by Gamble, Blackfan, and Hamilton (20) to result because of loss of base. The well known diuretic effect of salt-poor diets rests on this same basis. It may be stated empirically that loss or acquisition of body base is accompanied, except under the most extreme conditions, by loss or acquisition of an equivalent amount of water (21, 22). Pregnancy, on the other hand, in spite of the reduced concentration of serum electrolytes, is characterized by a positive water balance (56).

Furthermore, increased ammonia excretion may no longer be considered *prima facie* evidence of increased acid production and excretion. The urinary ammonia, as Gamble, Ross, and Tisdall

(23) have shown, is merely a device to spare body stores of base, and it does not reveal how the necessity for alkali conservation has arisen.   The increased ratio of ammonia nitrogen to total nitrogen excretion observed by Hasselbalch and Gammeltoft (27) and Slemons (56) in pregnancy may be merely an expression of the alkaline depletion which the authors have found.   An actual quantitative increase of ammonia excretion was not observed by those workers.   The increased ammonia nitrogen to total nitrogen ratio was due to a diminution of the denominator; ammonia excretion remained unchanged from the normal.   Moreover, the actual acidity of the urine in Hasselbalch and Gammeltoft's cases was less (pH 5.80) before delivery than after (pH 5.46).

In view of the facts set forth in the introduction and in the above discussion, the theory that the "acidosis of pregnancy" is the result of an unusual production of acid is untenable.

Hyperventilation.—On the basis of an increased serum alkalinity (a shift of pH from 7.30 to 7.45 in normals to 7.35 to 7.55 in pregnancy) found by Marrack and Boone (39), Austin and Cullen (1) attribute the reduction of reserve alkali to hyperventilation. These pH values were determined colorimetrically, a method which is open to criticism (Eisenman (16), Austin, Stadie, and Robinson (3)), especially when applied to pathological blood (unpublished studies from this laboratory).   At the alveolar CO_2 tensions existing before and after delivery, Hasselbalch and Gammeltoft (27) found no change of pH, the average value at both periods being 7.44, and furthermore neither Menten (40) nor Williamson (68) found a change of pH in pregnancy.   pH determinations on sera in pregnancy by methods of sufficient accuracy are as yet not available in the literature.

Were the reduction of reserve alkali, however, the result of hyperventilation, one would expect an electrolyte picture entirely different from that which is seen in pregnancy.   Peters, Bulger, Eisenman, and Lee (44) found that in hyperventilation, chronic (postencephalitic) or acute, reduced bicarbonate was compensated for by increased chlorides.   The concentration of total base always remained within normal limits, 152 to 160 mm.   This contrasts sharply with the unchanged chloride and lowered total base in pregnancy.

Hydremia in Pregnancy.—The idea that there is in pregnancy a

hydremia is supported by considerable evidence; namely, lowered specific gravity of the blood (42, 61, 69), decreased serum or plasma proteins (14, 17, 18, 34, 38, 47, 48, 69, and the authors), diminished freezing point depression (69), and increased water content and decreased total solids of the blood (60, 66, 69). But true dilution of the blood *in vivo*, except under extremely pathological conditions, is probably impossible. The findings of the authors do not lend support to a theory of blood dilution as an explanation for the diminution of serum proteins, bicarbonate, and total base. By plotting these constituents one against the other, no quantitative correlation can be detected. The percentage diminutions from the normal are widely different, those for serum protein and bicarbonate being about 16 per cent, for total base about 5 per cent. Chlorides, we have seen, show no change. Moreover, according to Plass and Matthew (48) the reduction of serum protein is chiefly at the expense of the albumin fraction, the globulin remaining essentially normal.

Probable Effect of Observed Changes.—No adequate explanation of the observed changes appears to be available. Yet the reduction of total electrolytes (of which total base may be considered the measure since acid cannot exist as such in the blood) and of serum protein must be accompanied by considerable reduction of osmotic pressure in the pregnant organism. Even though it is impossible to interpret the electrolyte or freezing point changes quantitatively in terms of osmotic pressure because of our ignorance of activity coefficients and other factors in media as complex as serum, the electrolyte changes here observed are, nevertheless, of the same order of magnitude as the freezing point depressions previously observed by Zangemeister (69).

Plass and Bogert (47), in considering this question are of the opinion that the reported changes in pH and the increase of fibrin which have been observed in pregnancy, probably prevent osmotic changes. The lack of proof of pH changes has been discussed. The decrease of albumin found by Plass and Matthew (48) is about 4 times as great as the increase of fibrin, whereas the minimal atomic weights of albumin and fibrin are 45,000 and 42,000 respectively (9), so that there is no basis for the assumption that the fibrin increase may compensate for the diminution of serum protein.

In view of the remarkable constancy of the concentration of serum electrolytes in normal individuals and the resistance, even in pathological conditions, offered by the body to fluctuations of that concentration (21–23, 43–45), the pregnant organism is unique in its ability to tolerate such changes without obvious symptomatology. This point of view may be emphasized by the following examples.

Moss (41) reports that when stokers and miners, who work at high temperatures and lose considerable body salts by excessive sweating, drink large quantities of water, they develop severe cramps. These can be prevented or relieved by ingestion of salt. In dogs a 15 per cent dilution of the blood produced by forced water intake was found by Greene and Rowntree (24) and Rowntree (52, 53) to result in convulsions, stupor, and eventually death. Intravenous injection of hypertonic saline would rapidly restore a moribund animal to normal, apparently none the worse for the experience. The peculiar diminution of serum electrolytes together with the reduction of alkaline reserve in pregnancy may be of significance as regards the delicate physiological balance existing in that condition. The violence and suddenness of the reactions of pregnant individuals to minor accidents, which are comparatively well sustained by the non-pregnant, is a matter of universal clinical experience.

Postpartum Changes.—It remains to point out the rapidity with which, following delivery, the normal blood picture is restored. Serum proteins were found by Plass and Matthew (48) to return to the normal level about the 3rd week after delivery; the alveolar CO_2 tension by Hasselbalch and Gammeltoft (27) by about the 15th day. In Case 66517 of the authors' series, by the 9th day serum bicarbonate had reached practically the normal level, serum proteins had increased 1 per cent, and total base had increased 3 mM. Cases 66436 and 67359, especially the former, show the same phenomena.

SUMMARY AND CONCLUSION.

1. Total electrolyte studies on the sera of twelve normal pregnant women and one non-pregnant woman, and serum total base determinations on ten normal non-pregnant individuals have been pre-

sented.   The values for the individual ions have been shown to be in close agreement with those of many other observers.

2. Inorganic phosphorus and chlorides in pregnancy show no changes from the normal.

3. There is a reduction of about 5 per cent (8 mM) of total base of the serum in pregnancy.   From the work of others this reduction would appear to be almost entirely at the expense of sodium.

4. There is a concomitant and equal reduction of the anion content of the serum in pregnancy.   This reduction is seen in serum protein, serum bicarbonate, and organic acid, chiefly in the latter two.

5. The term, "acidosis of pregnancy," as connoting an actual collection of abnormal acids in the blood is misleading.   On the contrary, the reduction of reserve alkali is associated with a diminution of base.

6. No satisfactory explanation for the observed changes is available.

7. The pregnant organism appears to have a unique ability to tolerate a reduced concentration of serum electrolytes.

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OXIDATION-REDUCTION SYSTEMS OF BIOLOGICAL SIGNIFICANCE.

II. REDUCING EFFECT OF CYSTEINE INDUCED BY FREE METALS.

By L. MICHAELIS and E. S. GUZMAN BARRON.

(From the Laboratory of Research Medicine, Medical Clinic, the Johns Hopkins University, Baltimore, and the Marine Biological Laboratories, Woods Hole.)

Part I. Oxygen Consumption of Cysteine.

1. Statement of Problem.—It has been shown (1) that the potential established by a cysteine solution at a metal electrode at a definite pH is the same whether platinum, or gold, or mercury was applied as an electrode provided no trace of oxygen was left in the solution. The speed with which the definite potential was reached was found to be much greater at the mercury than at the platinum or gold electrode. All of these electrodes were sensitive to oxygen in so far as the potential became more positive in the presence of small amounts of oxygen and besides became dependent on the rate of shaking or stirring. Here, too, mercury behaved differently in so far as the sensitivity towards oxygen, though easily detectable, was markedly less for mercury than for the two other metals. A suitable interpretation of the effect of oxygen was the assumption of a continuous chemical process taking place in the presence of oxygen which prevented the establishment of a true equilibrium. This process can only be an oxidation of cysteine. The effect of oxygen was not altered by the addition of KCN, so that it seemed unlikely that traces of iron salts were responsible for this oxidation. On the basis of these observations it occurred to the authors that the metal of the electrode itself might participate in this process.

2. Effect of Mercury.—The oxygen consumption of cysteine

was measured in Warburg's micro respiration apparatus with a manometer according to the principle of Barcroft. Every care was taken to work in practically complete absence of iron salts by the application of all those methods described by Warburg and his coworkers (2). 6 mg. of cysteine hydrochloride dissolved in 0.1 cc. of distilled water were placed in the small side vessel,
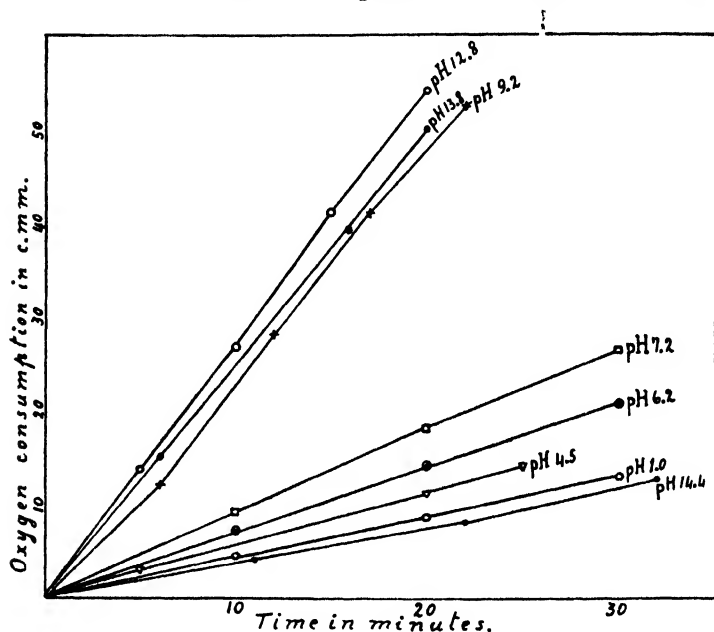
FIG. 1. Oxygen consumption of cysteine in presence of mercury. Total volume of the solution, V_F = 2.3 cc.; mercury, 0.2 cc. Buffers employed: for pH 14 to 12, NaOH; for pH 9.2, borate, prepared according to Warburg (2); for pH 6 to 7, phosphates; for pH 4.5, acetate; for pH 1, HCl. Temperature of the water bath for all experiments, 25.0° ± 0.03°.

while in the main vessel there was a buffer solution of definite pH and a small amount of mercury which had been purified by redistillation and by shaking with a solution of mercurous nitrate acidified with some drops of nitric acid. A control vessel without mercury was always used.

The common result of varied experiments of this kind was that

mercury proved to have a profound effect upon the oxidation by molecular oxygen. The curve (Fig. 1) showing the relation between pH and the rate of oxygen consumption in the case of mercury is very different from the one in which iron is used as catalyst. With iron salts there is a marked pH optimum at very slight alkaline reaction with a steep decrease towards both sides of the optimum, as Mathews and Walker (3) and Dixon and Tunnicliffe (4) have
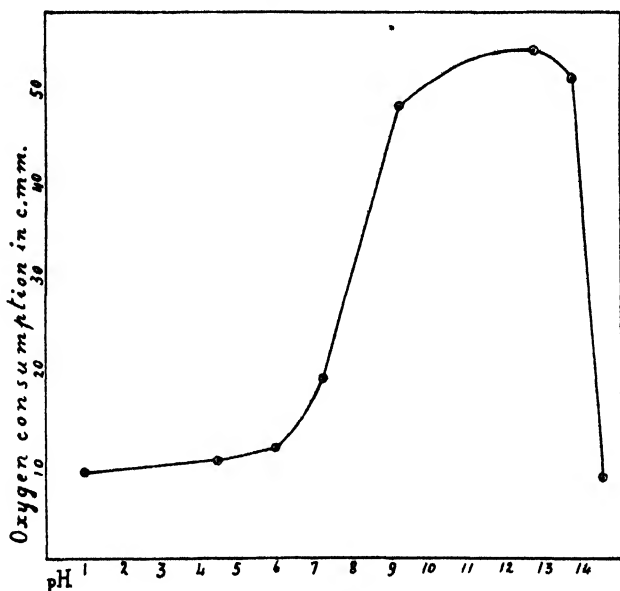
FIG. 2. The effect of pH on the rate of oxidation of cysteine in the presence of mercury. Initial velocity, *i.e.* oxygen consumption in the first 20 minutes in c. mm., plotted against pH.

shown. With mercury the maximum effect is obtained about at pH 12.8. With decreasing pH the oxidative effect approaches asymptotically a cert\in minimum value which however is still quite conspicuous at the lowest pH used in our experiments, pH 1. A further increase in pH beyond 12.8 rapidly depresses the activity of mercury (Figs. 1 to 3). But since the total electrolyte concentration in *e.g.* a 4 M solution of NaOH is very high, one cannot be sure whether this drop is due only to a change in pH.

For even at a pH of about 12.8, which is the optimum, the described effect of mercury is appreciably lower on addition of a
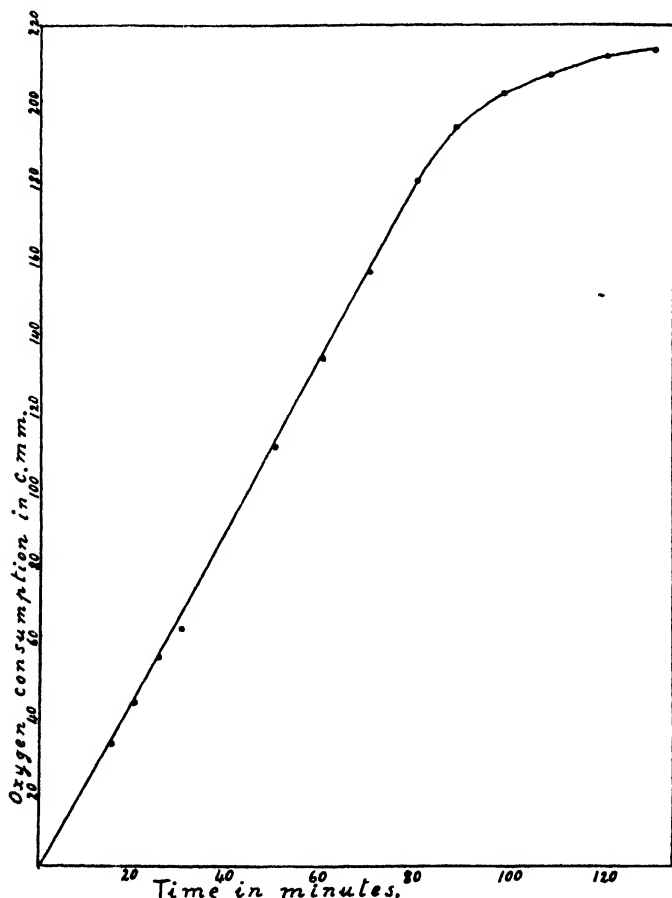
FIG. 3. Complete curve of oxygen consumption of cysteine with metallic mercury. Amount of cysteine used, 6 mg. at pH 12.8; mercury, 0.2 cc. Final consumption of oxygen: calculated 214.2, observed 213.8 c. mm. of oxygen.

neutral salt in very high concentration (Fig. 4). Considering only the range in which pH can be varied while the total salt content of the buffer is kept at a reasonably low value (not much

above 0.1 M), the curve is something like a dissociation curve with two asymptotes parallel to the abscissa and an approximate linear curve with a steep slope between the two asymptotes. The lower asymptote is, however, not at zero level, as it would be in a simple dissociation curve.

Some other experiments were carried out with a sheet of platinum amalgamated electrolytically in a mercurous nitrate solution
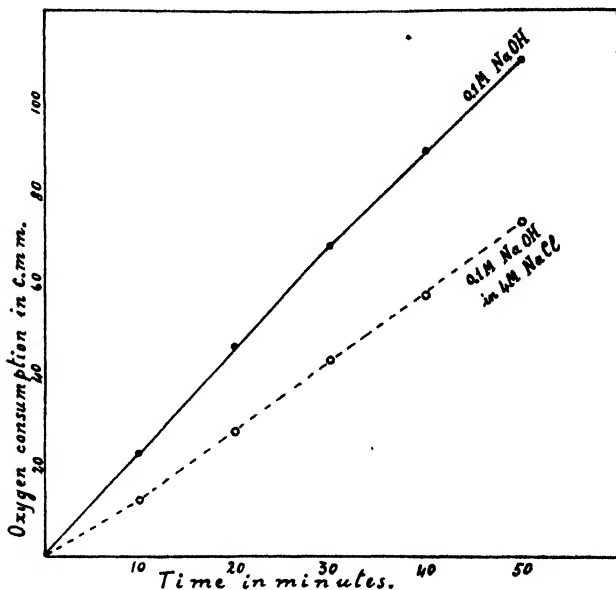
FIG. 4. Depressing effect of NaCl on the mercury action. Solid line indicates 0.1 M NaOH; dotted line, 0.1 M NaOH in 4 M NaCl.

in order to obtain a surface area of the metal comparable with the one used in the experiments to be described in the following part of this paper. No essential difference could be found apart from a generally lowered action in the case of amalgamated platinum, obviously due to the fact that the surface in the case of liquid mercury was greater.

The question arose as to whether this effect was due to the mercury metal or its ions dissolved in the solution. Addition of

mercurous nitrate or calomel or mercuric chloride instead of metallic mercury causes no O_2 consumption at all. As the solubility of the mercury salts is very small under the given conditions in presence of cysteine hydrochloride and the anions used in the buffer solutions, these experiments cannot directly decide whether or not there is a very slight effect of mercury ions which is un-
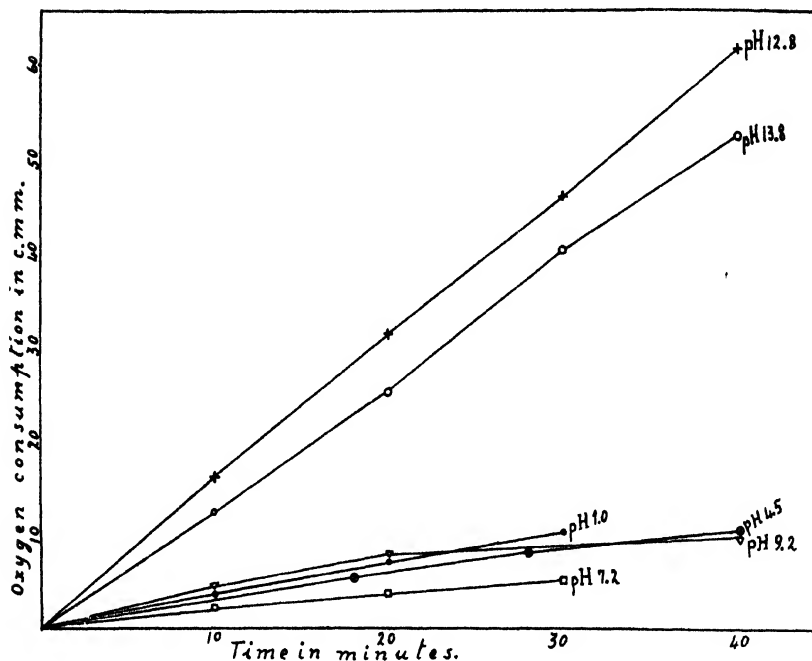
FIG. 5. The oxygen consumption of cysteine in the presence of platinized platinum. Each single experiment performed with a fresh platinization. Buffers used, same as in experiments with mercury.

measurably low under the conditions of the experiments, or none at all in an absolute sense. But the following two statements can be made. (1) The effect of the added mercury metal is not due to mercury ions which may have arisen by a slight oxidation of the metal. (2) If there be a catalytic effect of mercury ions at all, it must be much smaller than that of iron or copper ions, for these ions would have betrayed their presence even in

very minute concentrations. Anyhow, there is practically no catalytic effect of mercury ions at all, and in the foregoing experiments we have to deal with the effect of the metal surface itself.

It may be worth while adding that this effect, though due to a surface, is not altered by addition of what is called a surface-active substance such as ethyl- or phenylurethane.

3. Effect of Other Noble Metals.—Experiments of the same kind were carried out with sheets of bright and platinized platinum and with platinum plated with gold, silver, and mercury. The results with mercury-plated sheets have been mentioned already.
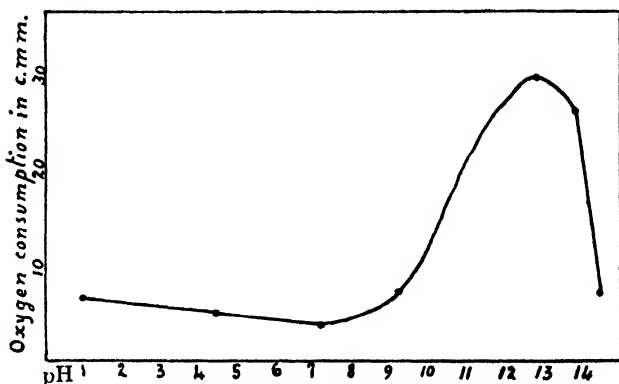
FIG. 6. The effect of pH on the rate of oxidation of cysteine in the presence of platinized platinum. Initial velocity, oxygen consumption in first 20 minutes, in c. mm.

At the gold surface an effect similar to mercury can be observed only to such an extent as to enable us to claim it to be just outside the range of any sources of error. The effect compared with a mercury-covered platinum sheet of the same area may be estimated about one-twentieth of the mercury effect. This effect could be observed with gold-plated platinum indeed, whereas a sheet of solid gold had no effect at all. With bright platinum, the effect is very small. The result of a single experiment may not always exceed the limits of error, but the agreement of all experiments renders it sure that there is a slight effect of blank platinum, too. But it is too slight to be studied quantitatively.

With platinized platinum there is a considerable oxidative effect. A somewhat durable platinization without addition of lead acetate to the platinum chloride solution can be obtained most usually only when a sheet of platinum has undergone the platinization process many times over a period of days or weeks. Preliminary daily platinizations were followed by a mild heating in the flame. Finally the black layer becomes more durable. But also this platinization loses its specific effect gradually, so it was not possible to carry out a series of experiments with one platinization. A duplicate of a single experiment showed often a loss of more than half of the original activity. However duplicates each with a fresh platinization obtained under similar conditions, agreed with each other fairly well, within 10 per cent or so. The following series is made with a fresh platinization for each experiment and the data plotted in Figs. 5 and 6 are average values of two experiments or more which agreed within the above stated limit. It can be seen that the dependence of the Pt effect is, in general, similar to that of Hg. There is an increasing effect with increasing pH, except for a steep decrease by increasing the pH beyond 13. There is one slight difference between the Pt and the Hg curve, consisting in the formation of a slight minimum around neutral reaction in the case of Pt. Calculations were always made with allowance for the very slight effect in the control without platinum due to traces of Fe salts. Except for the optimum range of pH with respect to the Fe catalysis, this correction was always negligible, and even within that range of pH it was but very slight.

Silver was used in the form of the crystalline precipitate as obtained at a platinum cathode from a strong solution of silver nitrate and in the form of a smooth deposit on platinum as obtained by electrolysis of silver cyanide. Neither form of silver showed any trace of catalysis. Less noble metals gave ambiguous results as they consume oxygen themselves under various conditions. But even allowing for this complication we think it probable that lead has a similar faculty, too, whereas lead salts have none.

4. *Effect of Cyanides.*—Since the early observations of Mathews and Walker (5) confirmed and explained afterwards by Warburg and Sakuma (2) it is well known that cyanides inhibit the catalytic

power of iron salts upon the oxidation of cysteine. In the case of noble metals the effect of cyanides is quite different. Thus with mercury (Fig. 7), KCN added at such concentrations as 0.01 M has no 'effect whatsoever on the oxygen consumption or on the reduction of methylene blue with which the next part of this paper is to deal. In fact all the experiments on the reduction of methylene blue by cysteine in presence of mercury were carried
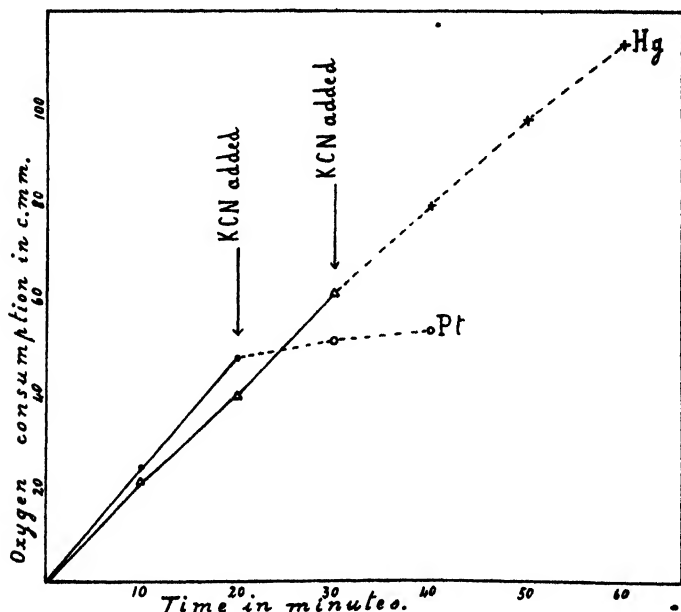
FIG. 7. The effect of cyanides on the action of mercury and platinized platinum upon the oxidation of cysteine. pH = 12.8; concentration of KCN, 0.01 M.

out by adding KCN to suppress any iron action. On the other hand, KCN has a strong inhibiting effect on the catalytic power of platinized platinum Platinum treated with KCN after removal of the cyanide by washing with distilled water, lost much more of its catalytic effect than could be expected from the spontaneous deterioration of the platinization. The effect of KCN therefore seems to be due to a decrease of the active surface area

by a slight corrosion of the uneven surface of the platinum black. The methylene blue reduction also is depressed by addition of KCN in the case of platinized platinum and it is entirely suppressed in a 0.01 M solution of KCN.
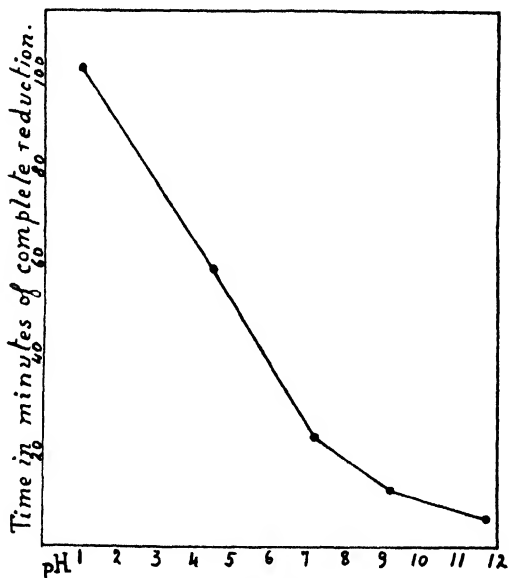
FIG. 8 Effect of pH on the time of complete reduction of methylene blue by cysteine in the presence of mercury. Temperature 22°. Final concentration of cysteine, 0.01 M; final concentration of methylene blue, 0 005 per cent; amount of mercury, 0.2 cc.

Part II. Reduction of Methylene Blue by Cysteine.

It has been described by Toda (6) in Warburg's laboratory that cysteine reduces methylene blue only in the presence of iron salts. These experiments could be easily confirmed. In solutions not absolutely free from iron the reduction of methylene blue by cysteine can be virtually eliminated by addition of a trace of KCN. So the problem arises in the reduction of methylene blue by cysteine as to whether iron salts can also be replaced by free metals.

Into Pyrex tubes of the same diameter were introduced 1 cc. of a 0.05 M solution of cysteine hydrochloride, 1 cc. of methylene blue solution (0.025 per cent), 0.2 cc. of the catalyst in the case of metallic mercury or ferrous sulfate solution, or a sheet of the metal in the other cases, and buffer to a final volume of 5 cc. A set of three tubes was used in each experiment connected by glass tubings through which a constant stream of hydrogen purified by heated platinized asbestos was passed, at 22'.

Both mercury and platinized platinum exhibited a strongly accelerating influence upon the reduction of methylene blue by cysteine. The rôle of pH is here the same as in the oxygen consumption experiments. The rate of reduction is extremely rapid at an alkaline range (pH 12), while it is slow at the acid side (Fig. 8). The rate of shaking plays a considerable part because the reaction goes on at an interface. In the case of liquid mercury there is an additional factor as the surface is increased by mechanical subdivision of the mass of the metal on shaking.

In an attempt to compare the efficiency of iron and mercury, we performed a series of experiments at pH 9.2 with borate buffer with the result that under these conditions 0.2 cc. of metallic mercury was found to be equivalent to 0.008 mg. of iron in the form of ferrous sulfate when the tubes are strongly shaken.

Gold-plated platinum, solid gold, and silver-plated platinum had no measurable catalytic effect on the reduction of methylene blue by cysteine.

<div style="text-align:center">CONCLUSION.</div>

In a solution of cysteine, O_2 consumption can be induced by some free metals. These metals are different from those which according to Mathews and Walker and Warburg and Sakuma work as catalysts in the form of their salts. Metals active in the form of their salts are chiefly Fe and Cu. Metals not active as salts but active in free metallic state are Hg, Pt, and Au. A metal neither active in the metallic state nor as salt is Ag. All of these catalyses are exhibited in the oxidation both by molecular oxygen and by methylene blue in absence of oxygen. Pt is very efficient in the form of platinum black, but much

less so in the form of bright or even gray platinum. Au shows only a small effect and only when electrolytically deposited on platinum. It seems quite inert in the form of solid gold.

KCN does not alter the effect of metallic mercury, but it inhibits strongly the action of platinum.

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ANIMAL CALORIMETRY.

THIRTY-EIGHTH PAPER.

THE SPECIFIC DYNAMIC ACTION OF MEAT IN HYPOPHYSECTO-MIZED DOGS.

BY OLIVER HENRY GAEBLER.

WITH THE TECHNICAL ASSISTANCE OF JAMES EVENDEN.

(From the Department of Physiology, Cornell University Medical College, New York City.)

A possible connection between pituitary function and the specific dynamic action of protein has been considered by several investigators (1–4). The results obtained by Foster (4) are particularly clear cut. In his experiments glycine administered by intraperitoneal injection increased heat production of the normal rat but not of the hypophysectomized rat. Kestner, Liebeschütz-Plaut, and Schadow (3) correlate lowered specific dynamic action in cases of Fröhlich's syndrome with a diminished rise in blood amino acids after meat ingestion when such cases are compared with the normal. The present experiments were carried out to determine whether any similar relationship could be observed in dogs before and after removal of the pituitary.[1]

The respiration calorimeter of this Department was used for the measurements of heat production. Total nitrogen of the urine was determined by the Kjeldahl-Gunning procedure, non-protein nitrogen of the blood by the method of Folin and Wu, and blood amino acids by the method of Folin (5). Blood samples were in all cases drawn from the jugular vein. In the case of the first animal used the necessary preliminary data on blood non-protein nitrogen, amino acid nitrogen, and heat production were obtained, but the animal did not survive the operation. The

[1] The operations were performed by Professor J. E. Sweet, of the Department of Experimental Surgery.

second animal was one which survived the operation, but irregularities in its behavior and the short time of its survival permitted only observations on the specific dynamic action of protein.

Control Experiment.

The animal used was Dog 59, a 10 kilo female bull-terrier. Thirteen respiration experiments were carried out and three experiments outside of the calorimeter in which blood amino acid and non-protein nitrogen and total nitrogen of the urine were determined. The animal received a standard diet of 100 gm. of cracker meal, 100 gm. of meat, 20 gm. of lard, and 10 gm. of bone ash, given at 4 p.m. and consumed before 5 p.m. The experiments were begun 15 to 17 hours later, after catheterizing the animal and discarding the urine. During the first period of several hours either the basal metabolism or the blood amino acid and non-protein nitrogen was determined. The animal was catheterized again and the urine collected. Beef heart, 200 gm., was given immediately thereafter. The portion was weighed out after cutting it and was warmed sufficiently to have an indifferent temperature to the hand. On calorimeter days the animal was then returned to the calorimeter and the heat production measured, beginning 1 hour after meat ingestion and continuing in hourly periods until 4 hours after giving meat. The mid points of the periods were therefore $1\frac{1}{2}$, $2\frac{1}{2}$, and $3\frac{1}{2}$ hours after the food had been taken. At the close of the experiment the animal was catheterized again and the urine collected.

The arrangement for the study of blood amino acids, blood non-protein nitrogen, and urine nitrogen was such that the urine was collected separately by catheter for each hour corresponding to a calorimeter period, and venepunctures were made during the basal period and $1\frac{1}{2}$, $2\frac{1}{2}$, and $3\frac{1}{2}$ hours after ingestion of 200 gm. of beef heart. The results of these determinations are given in Table I.

The animal did not survive the operation following these experiments sufficiently long to permit of similar observations. The results upon the normal animal are, however, of some importance in connection with the general problem. The average rise in heat production following ingestion of 200 gm. of beef heart is 4.42 calories per hour above the basal of 17.68 calories and is

virtually uniform for the 2nd, 3rd, and 4th hours following the meal.   During the same time blood non-protein nitrogen continues to rise, the increase above the basal level being twice as

TABLE I.

Data on Heat Production and Changes in Composition of Blood and Urine after Ingestion of 200 Gm. of Beef Heart.

Blood non-protein nitrogen and amino acid nitrogen in mg. per 100 cc., urine nitrogen in gm. per hour, heat in calories per hour.

		Time.		
Variable.	Basal period.	Hrs. after meat.		
		1 to 2	2 to 3	3 to 4
Blood non-protein N.	30.0	40.2	40.0	46.1
	33.9	40.0	41.1	50.0
	27.5	39.5	46.2	46.9
	30.5	39.9	42.4	47.7
Blood amino acid N.	7.3	9.8	9.1	9.3
	8.7	10.2	9.8	8.9
	7.2	9.1	8.6	8.1
	7.7	9.7	9.2	8.8
Total urine N.	0.148	0 387	0.478	0.532
	0.130	0.415	0.484	0.498
				0.586
	0.139	0.401	0.481	0.539
Heat (indirect).	17.84			
	18.12	23.18	21.59	21.38
	16.93	23.54	25.36	22.44
	18.56	21.29	21.03	23.95
	17.90	20.48	20.74	22.07
	16.18	20.69	22.53	21.33
	18.26			
	17.68	21.84	22.25	22.23

great during the last period as during the first.   The height of dynamic action of protein therefore does not appear to be more than qualitatively related to the concentration of products of protein digestion circulating in the systemic blood.   In the case

of the amino acid fraction of the non-protein nitrogen, as determined by the method used, the relationship is even less clear. This fraction increases only 2 mg. per 100 cc. of blood during the early period, and then falls again.   Factors, such as storage of products of protein digestion and resynthesis of amino acids, require further study before one can arrive at an interpretation. On the other hand, these observations in the normal dog must make one conservative in necessarily connecting changes in specific dynamic action and blood amino acid concentration in clinical cases.   Not the amino acid content of the blood, but the avidity with which tissue absorbs amino acid, converts it into urea, and passes this through the blood stream into the urine, parallels the intensity of the specific dynamic action.

Specific Dynamic Action after Removal of the Pituitary.

In a second experiment the increase in heat production following meat ingestion was measured in an animal which had survived removal of the pituitary.   The animal was Dog 60, a brown and white, long haired, 8.3 kilo female, operated upon April 19, 1928. There was a persistent diarrhea after the operation, and the food given between 4 and 5 p.m. was taken irregularly.   The animal, however, consumed the 200 gm. portions of beef heart given after determination of the basal metabolism a sufficient number of times to permit the measurement of the specific dynamic action.   A few days after two satisfactory experiments were completed a large ulcer developed which involved the left lower lip and gum; the animal became weak and refused all food.   It was killed and an autopsy performed on May 24, 1928.   Gross examination showed no trace of anterior or posterior lobes of the pituitary.   A specimen was taken of the brain in the region of the pituitary stalk. Serial sections of this part showed a small mass of cells approximately 160 by 110 by 80μ lying on the ventral surface and presenting the essential characteristics of pars intermedia tissue. Dr. J. F. Nonidez, of the Department of Anatomy, who kindly examined these preparations, reports that these cells probably represent a small fragment of the tip end of this part of the gland but that there is no evidence of hyperplasia of the cells.

The results of the experiments on this animal are given in Table II.   The fall in basal respiratory quotients on successive

days during the early part of the experiment marks the transfer of the animal from the stock ration to a diet of 400 gm. of beef heart, when it refused its food after the 1st day's experiment. Later on the basal respiratory quotients rose again to the original level when the animal's diet again contained carbohydrate. The basal respiratory quotients indicate that absence of the pituitary did not interfere with the oxidation of carbohydrate.

In both of the experiments after meat the animal remained perfectly quiet during the 2nd and 3rd hours, but during the

TABLE II.

Data on the Specific Dynamic Action after Removal of the Pituitary.

| Date. | Calories per hr. | | | R.Q. |
| | Basal. | Hrs. after 200 gm. beef heart. | | |
		2	3	
1928				
Apr. 30	15.26			0.86
May 1	16.48			0.76
" 1	15.50			0.73
" 1		18.52		0.75
" 1			18.25	0.74
" 10	14.43			0.85
" 10	14.88			0.85
" 12		18.47		0.78
" 12			18.45	0.78
" 14	14.62			0.81
	15.19	18.49	18.35	

4th hour became restless. The results for the 2nd and 3rd hours are, however, so uniform that they appear adequate. The basal heat production gradually fell, and the animal's weight decreased from 8.3 to 7.5 kilos during the experiment. The average basal heat production was 15.19 calories per hour, and the average of all periods after meat was 18.42 calories per hour, a rise of 3.23 calories per hour as compared with 4.42 calories in the 10 kilo control animal. The urine nitrogen per hour after meat, however, averaged only 0.233 gm. per hour, as compared with an average of 0.296 gm. per hour for all calorimeter experiments after meat

in the case of the control animal.[2] Hence discrepancies in the amount of protein catabolized probably account for the difference in the extent to which heat production was increased in the two animals.

Alcohol Checks.

In Table III the alcohol checks covering the entire time during which the experiments were in progress are recorded. Each result is the average of three hourly periods.   The low respiratory quotient on May 3 was traced to a leak in the residual system, which was repaired.   The heat values obtained on May 1 (Table II) are thus about 2 per cent too high.

TABLE III
Alcohol Checks.

Date	R Q average, three periods
1926	
Mar 13	0 665
" 23	0 664
Apr 12	0 657
May 3	0 654
" 7	0 660
" 29	0 665

CONCLUSIONS.

1. No marked change in specific dynamic action of protein was observed in a dog after removal of the pituitary.

2. The basal respiratory quotients of the animal did not indicate any change in oxidation of carbohydrate.

3. The height of specific dynamic action at a variable time after protein ingestion bears no quantitative relationship to the concentration of the amino acids which are circulating in the blood.

The author is indebted to Professor Lusk for helpful suggestions and criticisms.

[2] In Table I the average nitrogen per hour is higher for the same animal after ingestion of the same quantity of meat.   The urine in that case was collected hourly, while during calorimeter experiments it was collected for the entire time from ingestion of meat until some time after closing the last period.

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COMPARISON OF THE ANTIRACHITIC POTENCY OF ERGOSTEROL IRRADIATED BY ULTRA-VIOLET LIGHT AND BY EXPOSURE TO CATHODE RAYS.

By ARTHUR KNUDSON and CHESTER N. MOORE.

(*From the Laboratory of Biological Chemistry, Medical Department of Union University, Albany Medical College, Albany, and Research· Laboratory, General Electric Company, Schenectady.*)

Plate 1.

INTRODUCTION.

Previous reports (1, 2) from these laboratories have shown that antirachitic properties can be induced in various substances, such as cholesterol, yeast, cottonseed oil, *etc.*, by exposure to high voltage cathode rays (3). Experiments reported by Rosenheim and Webster (4) and Hess and Windaus (5) have indicated that the sterol, ergosterol, when irradiated by ultra-violet light is converted into a powerfully antirachitic substance, so that as small a dose as 0.0001 mg. of irradiated ergosterol per day cures or prevents rickets in rats kept on a rachitogenic diet. The recognition of this fact raises new problems of scientific interest, such as the optimum conditions for the production of the vitamin and the quantity required for the normal development of an animal of a particular species. Experiments on the antirachitic activity of ergosterol produced by exposure to cathode rays were therefore undertaken in order to determine the best procedure for obtaining the most potent product and to compare this potency with that obtained by ultra-violet irradiation.

EXPERIMENTAL.

Rats weighing from 35 to 60 gm. were put on the Steenbock rachitic diet, Ration 2965 (6), when they were 4 to 5 weeks old.

They were kept on this diet for 21 days at the end of which time they had usually developed a marked degree of rickets. x-Ray pictures of all the rats were taken before starting the experimental period. In all the rats bred in our laboratory a moderate to severe rickets is generally produced in 21 days. However, in a number of rats which were purchased from a supply house we found that a large number did not develop a marked degree of rickets until 2 or 3 weeks later. This fact would not have been evident at the beginning of the experimental period unless x-ray pictures had been taken at that time. The experimental period was continued for 21 days and a record of the food consumption during this period was kept. The rats that did not eat their food and lost weight during the experimental period were discarded to avoid any chance of healing effect being due to fasting. At the end of the experimental period x-ray pictures were again taken and these were compared with those taken at the beginning of the experimental period.

In judging the degree of healing we have designated the result as follows: "complete healing" when the epiphyseal region is almost closed up showing only a narrow cartilagenous area and the bone approaches its normal architecture; "advanced healing" when about two-thirds of the epiphyseal region is filled up with a deposit of calcium salts; "moderate healing" when a narrow band of Ca salts shows in the epiphyseal area; and "beginning healing" when there is a scattering of Ca salts which cast a hair-line shadow in the film

The line test was also carried out in all of these experiments in accordance with the procedure of Shipley (7). The degree of calcification was expressed as given above for the x-ray technique. In all the cases examined the x-ray has checked with the line test except that the line test is perhaps a little more sensitive and slight degrees of healing that are not visible with the radiograph are sometimes shown by the line test.

Recently Poulsson (8) has reported upon a method for the quantitative determination of vitamin D in which he emphasizes the value of the radiographic observation over the other methods of observation for the diagnosis and healing of rickets. We had come to a similar conclusion and in all of our work have placed greater confidence in radiographic observations because of the

TABLE I.

Effect of Cathode Rays on Antirachitic Activity of Ergosterol.

Experiment No.	Rat No.	Weight.	Cathode ray exposure of ergosterol.	Daily dose fed.	Degree of healing.	
					Radiographic.	Line test.
		gm.		*mg.*		
1	1200	59–77	In air 180 sec., 2 in. from	0.0125	Complete.	Complete.
	1201	77–81	window, 200 kv., 1 ma.	0.0125	"	"
	1202	57–71		0.0025	Moderate.	Moderate.
	1203	73–91		0 0025	"	"
	1204	76–101		0.0005	Beginning.	Beginning.
	1205	65–84		0.0005	None.	None.
	1206	67–81		0.0001	"	"
	1207	71–86		0.0001	"	"
2	1949	55–60	In air 30 sec., 2 in. from	0.0025	Advanced.	Advanced.
	1950	53–70	window, 200 kv., 1 ma.	0.0025	Moderate.	Moderate.
	1951	52–56		0.0005	None.	None.
	1952	51–56		0.0005	Beginning.	Beginning.
	1953	56–66		0.0001	None.	None.
	1954	51–61		0.0001	"	"
3	1208	74–96	In air 5 sec., 2 in. from	0 0125	Advanced.	Advanced.
	1209	57–70	window, 200 kv., 1 ma.	0.0125	"	"
	1210	65–79		0 0025	Beginning.	Beginning.
	1211	61–81		0 0025	"	"
	1212	59–79		0.0005	None.	None.
	1213	62–78		0.0005	"	"
	1214	58–77		0.0001	"	"
	1215	70–85		0.0001	"	"
	1232	63–80	Controls on rickets diet		None.	None.
	1240	50–35	+ 0.1 cc. olive oil daily.		"	"
4	1377	69–91	In air 1 sec., 2 in. from	0.0625	Advanced.	Advanced.
	1378	61–85	window, 200 kv., 1 ma.	0.0625	"	"
	1383	63–85		0.0125	None.	None.
	1384	65–80		0.0125	"	Beginning.
	1301	67–85		0 0025	"	"
	1302	64–80		0.0025	Beginning.	"
	1303	71–88		0.0005	None.	"
	1204	65–81		0.0005	"	"

TABLE I—*Continued.*

Experiment No.	Rat No.	Weight.	Cathode ray exposure of ergosterol.	Daily dose fed.	Degree of healing.	
					Radiographic.	Line test.
		gm.		*mg.*		
5	1385	59–70	In air 0.2 sec., 2 in. from window, 200 kv., 1 ma.	0.0312	Beginning.	Beginning.
	1386	59–79		0.0312	None.	None.
	1308	63–82		0.0062	"	"
	1362	60–66		0.0062	"	"
6	1274	69–85	In air at temperate of liquid air, 2 in. from .window, 30 sec., 200 kv., 1 ma.	0.0625	Complete.	Complete.
	1275	79–95		0 0625	"	"
	1276	63–75		0.0125	Advanced.	Advanced.
	1277	67–89		0 0125	Complete.	Complete.
	1278	69–87		0.0025	None.	None.
	1279	66–77		0.0025	"	"
	1280	65–78		0 0005	"	"
	1281	63–77		0.0005	"	"
7	1246	59–81	In atmosphere of N 30 sec., 2 in. from window, 180 kv., 1 ma.	0 0625	Complete.	Complete.
	1247	64–74		0 0625	"	"
	1248	64–86		0.0125	"	"
	1249	69–94		0 0125	"	"
	1184	75–91		0.0025	Moderate.	Moderate.
	1185	72–90		0 0025	"	"
	1186	71–89		0.0005	Beginning.	Beginning.
	1187	70–85		0.0005	"	"
	1188	72–85		0.0001	None.	None.
	1189	66–84		0.0001	"	"
8	1233	66–81	In N 30 sec., 2 in. from window, 180 kv., 1 ma. Special precaution to prevent oxidation.	0.0125	Advanced.	Advanced.
	1234	61–83		0.0125	"	"
	1235	59–73		0.0025	Moderate.	Moderate.
	1236	62–79		0.0025	"	"
	1237	61–72		0.0005	None.	None.
	1238	64–83		0.0005	"	"
	1242	55–55		0.0001	"	"
	1243	51–45		0.0001	"	"
9	1305	70–85	Dissolved in absolute alcohol and exposed 300 sec., 2 in., 180 kv., 1 ma.	0.0025	None.	None.
	1306	64–80		0.0025	"	"
	1307	62–71		0.0005	"	"
	1293	49–64		0.0005	"	"

TABLE I—*Concluded.*

Experiment No.	Rat No.	Weight.	Cathode ray exposure of ergosterol.	Daily dose fed.	Degree of healing.	
					Radiographic.	Line test.
		gm.		*mg.*		
10	1250	60–83	Dissolved in absolute al-	0.0625	Complete.	Complete.
	1251	57–79	cohol, and exposed in	0.0625	"	"
	1252	63–81	atmosphero of N 2 in.	0.0125	Advanced.	Advanced.
	1253	65–89	from window, 30 sec.,	0.0125	"	"
	1254	61–80	180 kv., 1 ma.	0.0025	None.	Beginning.
	1255	63–83		0.0025	"	"
	1256	54–67		0.0005	"	None.
	1257	67–79		0.0005	"	"
11	1393	59–80	Dissolved in olive oil and	0.0625	Complete.	Complete.
	1394	56–74	exposed in air 100	0.0625	"	"
	1395	67–93	sec., 1 in., 200 kv.,	0.0125	Moderate.	Moderate.
	1396	56–72	1 ma.	0.0125	"	"
	1397	66–73		0.0025	None.	None.
	1398	63–80		0.0025	"	"
	1399	57–56	Controls 0.1 cc. olive oil		None.	None.
	1400	58–67	daily.		"	"

fact that we had an x-ray picture at the beginning of the experimental period and that we could follow the healing effect by x-ray pictures at various intervals or at the end of the experimental period.

In our radiographic technique we have not found it necessary to resort to the use of an anesthetic as we have been able to cut down the time exposure to 0.2 to 0.3 of a second. The rat was tied down with legs outstretched and the lower half of the body only was exposed on one-half of a 5 × 7 superspeed x-ray film. The other half of the film was protected by a lead screen. In this way a 5 × 7 film served for two x-ray pictures. We have found that two dozen pictures can be taken and developed in about 2 hours time.

The x-ray outfit used in making radiographs of the rats consisted essentially of a high tension transformer with autotransformer control, filament transformer and control, time-switch, meters, and Coolidge 30 Ma. Radiator Type x-ray tube. The

outfit was adjusted to operate at 40 milliamperes and 56,000 volts (maximum). With a distance of 10 inches from focal spot to film, exposures of about 0.3 second were sufficient for making radiographs of the rats on Eastman Duplitized x-ray films.

The ergosterol (m p. 152°) used in all of our tests was obtained from the Eastman Kodak Company Laboratories[1] and was prepared from ergot. The ergosterol in ergot is accompanied by a small proportion of a sterol called fungisterol from which the ergosterol may not be entirely free. After irradiation of the material it was dissolved in ether or if already dissolved in alcohol as it was in some cases during irradiation this was added to the olive oil and the alcohol or ether distilled off under reduced pressure below a temperature of 60°. The test preparation dissolved in olive oil was then administered to rats for a period of 21 days.

Effect of Cathode Ray Exposure on the Activation of Ergosterol.

The tube recently developed by Coolidge (3) for the production of high voltage cathode rays outside of the generating tube was used in all of these experiments with cathode rays.

Experiment 1.—5 mg. of ergosterol were spread out on a 3 inch Petri dish in a thin film by means of ether and exposed to cathode rays in air for *180 seconds*, 2 inches from the window of the tube operating at 200,000 volts and 1 milliampere. This preparation and those of the subsequent experiments were fed to rachitic rats daily in doses that are indicated in Table I. The results of these tests along with the other experiments on cathode ray exposure are shown in Table I.

Experiments 2, 3, 4, and 5.—These experiments were similar to Experiment 1, except that the cathode ray exposures were 30 seconds, 5 seconds, 1 second, and 0.2 second respectively.

Experiment 6.—5 mg. of ergosterol were spread out in a thin film on the bottom of a tin dish $1\frac{1}{2}$ inches in diameter. The bottom of this dish was sealed to a chamber of the same diameter which had a $\frac{3}{16}$ inch tube on each side so that the chamber could be filled with liquid air in a layer about $\frac{3}{4}$ inch thick. In this way the ergosterol was kept at approximately the temperature of liquid

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[1] We are indebted to Dr. C. E. K. Mees of the Eastman Kodak Company for our first lot of ergosterol used in this research work.

air ($-180°$) during the exposure to cathode rays. It was exposed in air at a distance of 1 to 2 inches from the tube for 30 seconds, at 200,000 volts and 1 milliampere.

Experiment 7.—5 mg. of ergosterol were spread out on a 3 inch Petri dish in a thin film by means of ether and exposed to cathode rays in an atmosphere of pure nitrogen for 30 seconds, at a distance of 2 inches from the window of the tube operating at 180,000 volts and 1 milliampere.

Experiment 8.—5 mg. of ergosterol were exposed to cathode rays for 30 seconds in an atmosphere of pure nitrogen with 180,000 volts and 1 milliampere, 2 inches from the window. The olive oil in this experiment in which the irradiated ergosterol was dissolved was freed from all traces of oxygen by heating it to 200° and passing pure nitrogen gas continuously through it during the heating and subsequent cooling. After dissolving the material in the olive oil it was put in the refrigerator and taken out only during the feeding period. In this way the chances for oxidation of the material were cut down to a minimum.

Experiment 9.—In this experiment the ergosterol was dissolved in absolute alcohol (1 mg. per 5 cc.) and exposed to cathode rays in alcohol solution, which was about 2 to 3 mm. thick. The exposure was made in an atmosphere of pure nitrogen, at a distance of 2 inches from the window, for 300 seconds at 180,000 volts and 1 milliampere.

Experiment 10.—This experiment was similar to Experiment 8 except that exposure was 30 seconds instead of 300.

Experiment 11.—In this experiment the ergosterol was first dissolved in olive oil (5 mg. per 0.1 cc. of oil) and spread out on a dish 2 inches in diameter and exposed to cathode rays in air for 100 seconds, 1 to 2 inches from the window of the tube operating at 200,000 volts and 1 milliampere.

In the experiments with cathode ray exposure the most potent product was obtained in Experiment 7 with an exposure of 30 seconds in an atmosphere of pure nitrogen gas. This preparation brought about a distinct healing effect with 0.0005 mg. per day. Experiment 8 was similar to Experiment 7 except that special precautions were taken throughout the whole experiment to prevent any oxidation. This preparation, however, was apparently not quite as potent as it did not show any healing **with**

0.0005 mg. Since the activation of the ergosterol in both of these experiments was carried out in an atmosphere of pure nitrogen gas, the synthesis of vitamin D does not seem to be an oxidation reaction.

The preparation of Experiment 1 was of about the same potency as that from Experiment 7. The exposure in air which was 6 times longer than in nitrogen would indicate that the formation of vitamin D is not affected by the presence of oxygen. An exposure of 30 seconds in air gave about the same potency as an exposure of 180 seconds but exposures of 5 seconds, 1 second, and 0.2 of a second respectively brought about the formation of products of less potency.

In the experiments where ergosterol was dissolved in absolute alcohol and in olive oil before exposure to cathode rays, less potent products were obtained than when exposed in a dry form.

Exposure of the ergosterol at approximately the temperature of liquid air also formed a product of less antirachitic action than exposure at room temperature. The fact that at lower temperature a less potent product is formed is similar to results reported by Bills and Brickwedde (9) who found that cholesterol exposed to ultra-violet light at liquid oxygen temperature was less potent than at room temperature. Webster and Bourdillon (10) have also reported a similar behavior with ergosterol exposed to ultraviolet light at lower temperatures.

Effect of Irradiation by Ultra-Violet Light on the Activation of Ergosterol.

In all of the experiments with ultra-violet light a Cooper Hewitt mercury vapor quartz lamp operating at 5 amperes and 140 volts on a 220 volt direct current circuit was used. A solution of ergosterol dissolved in absolute alcohol (1 mg. for 5 cc.) was used in most of the experiments with ultra-violet light and this solution was irradiated in a quartz cell 1 cm. thick.

Experiment 1.—The alcoholic solution was irradiated for 300 minutes, 18 cm. from the lamp. The preparation was fed to rachitic rats in doses that are indicated in Table II. The results of this experiment and subsequent ones with ultra-violet irradiation are given in Table II.

Experiments 2, 3, 4, and 5.—These experiments were similar to

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TABLE II.

Effect of Ultra-Violet Radiation on Antirachitic Activity of Ergosterol.

Experiment No.	Rat No.	Weight.	Ultra-violet irradiation of ergosterol.	Daily dose fed.	Degree of healing.	
					Radiographic.	Line test.
		gm.		*mg.*		
1	1412	60–81	Irradiated in quartz cell in alcohol solution, 300 min. at 18 cm.	0.0125	None.	None.
	1413	61–79		0.0125	"	"
	1414	62–77		0.0025	"	"
	1415	58–65		0.0025	"	. "
	1416	53–73		0.0005	"	"
	1417	56–72		0.0005	"	"
2	1191	67–86	Alcoholic solution irradiated 30 min. at 18 cm.	0 0125	Complete.	Complete.
	1192	63–67		0.0125	"	"
	1193	55–60		0.0025	"	"
	1194	61–70		0 0025	Advanced.	Advanced.
	1196	55–50		0.0005	"	"
	1197	51–59		0.0005	"	"
	1198	61–69		0.0001	Beginning.	Beginning.
	1199	59–57		0.0001	"	"
3	1315	66–93	Irradiated 3 min. at 18 cm.	0.0025	Complete.	Complete.
	1333	79–103		0.0025	"	"
	1347	79–90		0.0005	"	"
	1349	78–94		0.0005	"	"
	1350	73–92		0.0001	Advanced.	Advanced.
	1351	73–92		0.0001	"	"
	1461	45–58		0.0001	"	"
	1462	46–52		0.0001	"	"
	1421	71–94		0 00002	Beginning.	Beginning.
	1422	74–104		0.00002	"	"
	1463	42–55		0.00002	"	"
	1464	108–130		0.00002	None.	"
	1465	75–91		0.00002	Beginning.	"
4	1370	64–66	Alcoholic solution irradiated 15 sec. at 18 cm.	0 0025	Complete.	Complete.
	1371	47–39		0 0025	"	"
	1372	59–62		0.0005	"	"
	1373	56–65		0 0005	"	"
	1374	55–65		0 0001	Advanced.	Advanced.
	1375	49–44		0.0001	"	"
	1435	75–89		0 00002	None.	Beginning.
	1436	78–89		0.00002	"	"

TABLE II—*Concluded*.

Experiment No	Rat No	Weight	Ultra-violet irradiation of ergosterol	Daily dose fed	Degree of healing.	
					Radiographic	Line test
		gm		*mg*		
5	1445	60-73	Alcoholic solution irra-	0 0125	Complete.	Complete.
	1447	59-74	diated 1 sec at 18	0 0125	"	"
	1449	49-64	cm.	0 0025	"	"
	1450	49-62		0 0025	"	"
	1451	48-64		0 0005	Advanced.	Advanced.
	1452	49-67		0 0005	"	"
	1453	49-67		0 0001	None.	None.
	1454	46-57		0 0001	Beginning	Beginning.
6	1224	63-83	Alcoholic solution irra-	0 0125	Complete	Complete.
	1225	63-84	diated 30 min., as in	0 0125	"	"
	1226	61-78	Experiment 2 except	0 0025	"	"
	1227	60-74	for special precaution	0 0025	"	"
	1228	56-67	to check oxidation	0 0005	Advanced	Advanced.
	1229	68-93		0 0005	"	"
	1230	65-79		0 0001	Beginning.	Beginning.
	1231	60-73		0 0001	"	"
7	1387	57-70	Irradiated in dry form	0 0625	Complete	Complete.
	1388	70-95	in thin film, 30 min	0 0625	"	"
	1389	67-88	at 18 cm.	0 0125	"	"
	1390	66-86		0 0125	"	"
	1391	70-93		0 0025	"	"
	1392	66-71		0 0025	"	"
	1477	55-50		0 0005	Moderate	Moderate.
	1478	40-35		0 0005	"	"
	1479	48-57		0 0001	None.	None.
	1480	43-49		0 0001	"	"
	1455	49-61	Untreated ergosterol,	0 0625	None.	None.
	1456	50-63	as control.	0 0625	"	"
8	1366	87-102	Dissolved in olive oil	0 0025	Beginning.	Beginning.
	1368	84-102	(5 mg. for 0.1 cc.) and	0 0025	"	"
	1379	73-88	irradiated 30 min. at	0 0005	None.	"
	1380	66-82	18 cm.	0 0005	"	None.
	1381	68-81		0 0001	"	"
	1382	67-83		0 0001	"	"

Experiment 1 except that the irradiations were 30 minutes, 3 minutes, 15 seconds, and 1 second respectively.

Experiment 6.—The ergosterol solution was given the same exposure as in Experiment 2 and the same special precautions were taken to check oxidation as was done in Experiment 8 on the effect of cathode rays.

Experiment 7.—5 mg. of ergosterol were spread out in a thin film by means of ether on a $2\frac{1}{2}$ inch watch crystal and after evaporation of the ether, irradiated in dry form for 30 minutes at 18 cm.

Experiment 8.—5 mg. of ergosterol were dissolved in 0.1 cc. of olive oil and this was spread out on a 2 inch watch-glass and irradiated for 30 minutes at 18 cm.

In the experiments with ultra-violet irradiation of ergosterol it will be noted that an exposure for 30 minutes gives a preparation quite potent with a dose of 0.0001 mg. per day. It is also interesting to note that in Experiment 7 where special precautions were taken to cut down processes of oxidation no greater potency was obtained. An exposure of 15 seconds and 3 minutes gave products that were somewhat more potent than an exposure of 30 minutes, showing a healing effect with as low a dose as 0.00002 mg. per day. Even an exposure of 1 second produced a marked anti-rachitic action in the ergosterol. On the other hand, irradiation of ergosterol solution for 300 minutes showed no healing effect with as large a dose as 0.0125 mg., as though the greater part of the antirachitic activity was destroyed with such prolonged irradiation. Ergosterol irradiated in dry form and in olive oil solution is not rendered as potent as when irradiated in an alcoholic solution. This result may perhaps be due to the absorption of the ultra-violet energy by the olive oil and by the crystals of ergosterol at the surface of the film.

From these two sets of experiments we find that the lowest dose which brings about a healing effect of rickets by cathode ray exposure is 0.0005 mg. per day while with ultra-violet light a dose as low as 0.00002 mg. is effective. Thus, under the various conditions studied, a more potent product is obtained by ultra-violet irradiation than by cathode ray treatment, and irradiation with the mercury vapor quartz lamp of 15 seconds to 3 minutes is more effective than the longer exposure of 30 minutes which has been more or less generally used by a number of investigators.

*Effect of Combined Cathode Ray Exposure and Ultra-Violet
Irradiation on the Activity of Ergosterol.*

Since a more potent product is obtained by ultra-violet irradiation it was thought it might be of interest to see whether subse-

TABLE III.

*Effect of Combined Cathode Ray Exposure and Ultra-Violet Irradiation on
Activity of Ergosterol.*

Experiment No.	Rat No.	Weight.	Treatment of ergosterol.	Daily dose fed.	Degree of healing.	
					Radiographic.	Line test.
		gm.		*mg.*		
1	1403	61–80	Irradiated in quartz cell	0.0125	Advanced.	Advanced.
	1404	65–90	in alcohol solution 30	0.0125	Complete.	Complete.
	1405	70–94	min., 18 cm.; then evap-	0.0025	Advanced.	Advanced.
	1406	65–67	orated and exposed to	0.0025	"	"
	1407	65–82	cathode rays in dry	0.0005	None.	None.
	1408	70–70	form 30 sec., 2 in.,	0 0005	Beginning.	Beginning.
	1354	89–116	250 kv., 1 ma.	0.0001	None.	None.
	1355	79–99		0.0001	"	"
2	1356	74–94	Irradiated in quartz cell	0 0025	Complete.	Complete.
	1357	90–110	in alcohol solution 30	0 0025	"	"
	1358	86–104	min., 18 cm. and then	0.0005	"	"
	1359	83–101	evaporated to dryness	0.0005	"	"
	1360	77–101	(control for Experi-	0 0001	Beginning.	Beginning.
	1363	81–104	ment 1).	0.0001	"	"
3	1656	50–61	Exposed to cathode rays	0.0125	Complete.	Complete.
	1657	54–67	in dry form 30 sec., 2	0.0125	"	"
	1658	52–59	in. from window, 200	0.0025	"	"
	1659	49–54	kv., 1 ma., and taken up	0.0025	"	"
	1660	55–67	in alcohol and irradi-	0.0005	Advanced.	Advanced.
	1661	55–66	ated in quartz cell.	0 0005	"	"
	1662	53–62	Ultra-violet lamp 3	0.0001	Beginning.	Beginning.
	1663	49–55	min. at 18 cm.	0.0001	"	"

quent cathode ray treatment has any effect on the potency. It would also be of interest to note what effect ultra-violet irradiation has after cathode ray exposure.

Experiment 1.—An alcoholic solution of ergosterol was irradiated with a mercury vapor quartz lamp for 30 minutes at 18 cm.

The solution was then evaporated on a water bath and the residue taken up with ether and the ether solution evaporated on a 3 inch watch crystal. The irradiated ergosterol was in this manner spread out on the watch crystal in a thin film (3 mg.). It was then exposed to cathode rays for 30 seconds, at a distance of 2 inches from the window of the tube operating at 200,000 volts and 1 milliampere. The details as to the dosage fed and the results of the experiment and also of Experiments 2 and 3 below are shown in Table III.

Experiment 2.—This was carried out as a control on Experiment 1. The ergosterol was given the same treatment with ultra-violet irradiation, evaporation of the solution, and taking up with ether, but was not exposed to cathode rays.

Experiment 3.—In this experiment ergosterol was first exposed to cathode rays in dry form spread out in a thin film on a watch crystal. Exposure was for 30 seconds, 2 inches from the window of the tube operating at 200,000 volts and 1 milliampere. It was then dissolved in absolute alcohol and irradiated with the mercury vapor lamp for 30 minutes at a distance of 18 cm.

The preparation which was irradiated with ultra-violet light and then subsequently exposed to cathode rays is less potent than the control experiment with only ultra-violet irradiation. It shows about the same potency as is obtained with cathode ray exposure. The fact that the product is less potent would indicate that cathode rays have a destructive action on vitamin D as well as a synthetic reaction for it. The destructive action of cathode rays on vitamin D is apparently much more rapid than with ultra-violet irradiation.

In Experiment 3 the potency of the product is the same as with ultra-violet irradiation alone. The preliminary cathode ray exposure does not apparently act on all of the provitamin so that subsequent ultra-violet irradiation can form more vitamin D.

Spectroscopic Observation.

The absorption spectrum of ergosterol was photographically recorded by means of a Hilger quartz spectrograph with a mercury vapor quartz lamp as a source of light. A quartz cell 10 mm. in width was used with pure ether as a solvent.

The absorption of ultra-violet light by ergosterol was found to

be considerable in a solution of 1:5000 (Fig. 1, Exposure 2) particularly in the region from 3000 Å. down to 2500 Å. After

Effect of Cathode Ray Exposure on Antirachitic Activity of Substances When Exposed behind a Quartz Plate

Experiment No.	Rat No	Weight.	Treatment of substance fed.	Daily dose fed	Degree of healing	
					Radiographic.	Line test.
		gm		*mg.*		
1	998	69–88	Commercial cholesterol exposed to cathode rays behind quartz plate 30 sec , 1 in , 200 kv., 1 ma	3	None.	None.
	999	72 -99		3	"	"
	996	58–66	Controls, same exposure without quartz plate.	3	Complete.	Complete.
	997	65- 85		3	"	"
2	1052	50–49	Dry yeast exposed to cathode rays behind quartz plate, same exposure as Experiment 1	10	None.	None.
	1053	55–65		10	"	"
	1054	59–56		10	"	"
	1055	53–53		10	"	"
	1088	70–81	Controls, same exposure without quartz plate	1	Advanced.	Advanced.
	1089	66–77		1	Complete.	Complete.
	1090	64–75		1	"	"
	1091	69–78		1	Advanced.	Advanced.
3	1577	61–68	Ergosterol, exposed to cathode rays behind quartz plate, same exposure as Experiment 1.	0 0625	None.	None.
	1578	53–50		0 0625	"	"
4	1589	46–45	Ergosterol, exposed to cathode rays behind quartz plate, film of ergosterol also on plate nearest to window of tube.	0 0625	None.	None.
	1590	65–57		0 0625	"	"

irradiation with ultra-violet light, there is a disappearance of the characteristic absorption bands. In Fig. 1, Exposure 3, is shown the change in absorption bands of ergosterol (1:5000) after 5

hours irradiation in dry form with the mercury vapor quartz lamp. The irradiated ergosterol solution transmits considerably more ultra-violet energy than the non-irradiated ergosterol. A similar change in the absorption spectrum of ergosterol occurs after exposure to cathode rays. In Fig. 1, Exposure 4, is shown the absorption spectrum of ergosterol which has been exposed to cathode rays 150 seconds, 1 inch from the window of the tube operating at 250,000 volts and 1 milliampere.

Experimental Evidence That the Effect of Cathode Rays on Ergosterol Is Not Due to the Production of Ultra-Violet Light.

The absorption spectra of ergosterol irradiated by ultra-violet light and by exposure to cathode rays are similar and the question arose as to whether cathode rays produce their action through the production of ultra-violet light. This point was tested out early in our work with yeast and cholesterol, and later with ergosterol. In the experiments with these products they were spread out in a thin film on a watch crystal which was sealed with paraffin to the back of a quartz plate. The front of the quartz plate was then bombarded by cathode rays and the yeast, cholesterol, or ergosterol could then be affected only by radiation through the quartz plate. By sealing the watch crystal to the quartz plate the substances were protected from bombardment of high speed electrons coming around to the rear of the quartz plate opposite the window of the tube and also on the opposite side as prepared above. This was done to see whether the cathode rays impinging on the ergosterol could produce sufficient ultra-violet energy at the point of impact to pass through the quartz plate and activate the ergosterol on the opposite side. The results of these experiments are given in Table IV.

Yeast, cholesterol, and ergosterol were not activated when exposed to cathode rays behind a quartz plate. These experiments indicate, therefore, that the antirachitic properties produced by cathode rays are not due to exposure to ultra-violet light produced by the rays themselves.

SUMMARY.

1. Ergosterol exposed to cathode rays with the tube operating at 180,000 to 200,000 volts is not rendered as potent as when

irradiated with ultra-violet light from a mercury vapor quartz lamp.

2. The highest potency that we obtained by cathode ray exposure was 0.0005 mg. per day and by ultra-violet irradiation 0.00002 mg. per day.

3. Ergosterol exposed to ultra-violet light for 15 seconds was more potent than that exposed for 30 minutes.

4. Ergosterol exposed to cathode rays undergoes a similar change in the absorption spectra as when exposed to ultra-violet light.

5. The manner in which cathode rays produce their antirachitic action does not seem to be due to the production of ultra-violet light.

The authors gratefully acknowledge the encouragement and assistance of Dr. W D. Coolidge throughout the progress of the work.

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EXPLANATION OF PLATE 1.

FIG. 1. Absorption spectra with Hilger quartz spectrograph.
Exposure 1.   Ether in quartz cell
Exposure 2.   Ether solution of ergosterol (1 mg per 5 cc).
Exposure 3.   Ether solution of ergosterol irradiated in dry form with mercury vapor quartz lamp for 5 hours.
Exposure 4.   Ether solution of ergosterol exposed to cathode rays in dry form for 150 seconds, 2 inches from window of tube operating at 250 kilovolts, 1 milliampere.
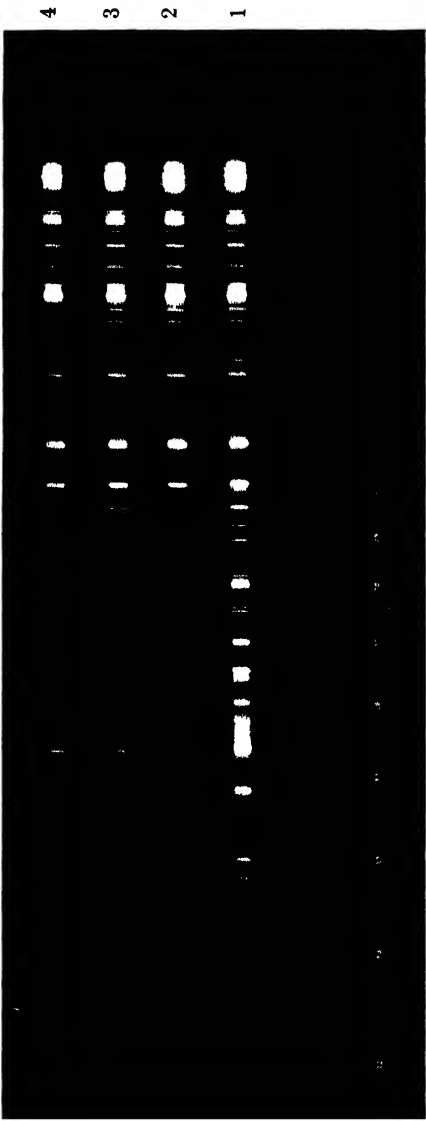
FIG. 1

THE TETANY OF FASTING IN EXPERIMENTAL RICKETS.

By T. S WILDER.

(From the Laboratory of the Department af Pediatrics, the Harvard Medical School, and the Medical Division of the Children's Hospital, Boston.)

An unusual type of experimental tetany produced by fasting rachitic rats was described by Cavins in 1924 (1). This author reported blood calcium values as low as 5.4 and an elevation of the inorganic phosphate to 16 mg. in animals killed after 2 days without food. When allowed to fast for 4 days one rat died in convulsions. Histological sections from such animals revealed the presence of bone healing as was also noted, in 1922, by McCollum, Simmonds, Shipley, and Park (2).

Repeating the above experiments we have obtained a similar elevation of the blood phosphate accompanied by severe tetanic convulsions, and in animals which survived a few days have been able by x-ray to demonstrate healing of long bones. Normal rats when fasted did not reproduce these phenomena.

The mechanism of this type of tetany appeared to us worthy of investigation. Our measurements on the blood showed such a great elevation of the inorganic phosphate during fasting and so slight a depression of the calcium that we assumed that the change in the phosphate factor was the primary event. Since our experimental animals were on a nearly zero phosphorus intake, it was necessary to postulate a sudden liberation of endogenous inorganic phosphate into circulation. That this high blood phosphate caused a secondary lowering of the calcium seemed reasonable since Salvesen (3) obtained a like effect when he fed large amounts of phosphate to dogs, as also did Binger (4) when he injected phosphate into experimental animals by the intravenous route. In 1927, Karelitz and Shohl (5) also observed a similar phenomenon after adding phosphate to the rickets-producing diet of rachitic rats, though it is noteworthy in this connection that

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their animals refused the phosphate diet to the extent of considerable loss in weight. As we shall show, the type of results reported by these last authors can be produced by fasting rachitic animals. This fact casts doubt on the supposition of Karelitz and Shohl that ingestion by rachitic rats of large amounts of phosphate effects fundamental changes in methods of phosphorus absorption and excretion. That is to say, no allowance was made in their experiments, otherwise very conclusively

TABLE I

Chief Forms of Phosphate of Whole Blood in Mg per 100 Cc

	Group No	In-organic P * (1)	Acid-soluble P * (2)	Total P * (3)	Ester P † (2)−(1)	Lipoid P † (3)−(2)
Rachitic rats (non-fasting controls)	1	2 4	23 3	34 2	20 9	10 9
	2	2 5		34 1		
	3	2 9	23 9	36 3	21 0	12 4
	4	2 5	21 1	32 5	18 6	11 4
Average		2 6	22 8	34 3	20 3	11 6
Rachitic rats (fasted and in convulsions).	1	9 5		46 6		
	2	9 3	33 3	40 1	24 0	6 8
	3	8 2	36 0	46 8	27 8	10 8
	4	9 3	38 5	45 7	29 2	7 2
Average		9 1	35 9	44 8	27 0	8 3
Rachitic rats (fasted, then fed; i e , convulsions, and recovery).		2 7	21 6	36 2	18 9	14 6

* Measured.
† Calculated

worked out, for the large quantity of phosphorus released from body tissues during fasting.

It was in consequence of these various but evidently related findings that we have directed our attention chiefly toward the source of phosphate in our fasting animals. Speculation on the probable endogenous source of phosphate indicated as possibilities: (a) the red blood corpuscles, known to be rich in organic and lipoid phosphate, and (b) the protoplasm of the body, especially that of the skeletal musculature.

In the former event some of the bound phosphate of the red cells would be released as inorganic phosphate. This seemed a good possibility and to test it measurements were made of the various phosphorus fractions of the whole blood, first on rachitic rats, then on fasted rats (in convulsions), and finally on rachitic rats that had been fasted and then given food again. Assuming that the total phosphorus of the whole blood is the sum of three principal fractions which may be termed the "inorganic," the "ester," and "lipoid" phosphate respectively, we find that there is a definite increase in inorganic phosphate in our fasted rats while the ester and lipoid fractions remain undepleted; indeed, there is actually an appreciable increase in the values for these fractions (see Table I). In other words no phosphate has left the cells; we may therefore assume that the excess inorganic P has entered the blood stream from body tissues outside the circulation.

Further evidence that there is an exogenous source of phosphorus flooding the blood stream during fasting is supplied by a few measurements, on the fourth group of fasted rats, which are not included in Table I. It may be seen from Table I that in this group the inorganic P of the *whole blood* was 9.3. The inorganic P of the *serum* was found to be 13.5, which, with a hematocrit of 44.5, supplies a value of 4.1 for the *cells*. The serum value was, therefore, over 3 times the cell value which is contrary to the usual close agreement of within 1 to 2 mg. A reasonable explanation for this discrepancy would seem to be that the flood of inorganic phosphorus into the serum from exogenous sources has been so sudden that equilibrium between serum and cells has not yet been established. This surmise would also explain the difference between the high *serum* values obtained by Cavins and also by us (15 to 16 mg.), and the much lower *whole blood* values listed in Table I, and averaging 9.1 mg.

It is of decided incidental interest in connection with the above, that on refeeding rachitic rats after a fast, all partition values return to the rachitic levels.

Muscle protoplasm contains, as compared with blood plasma, a relatively enormous concentration of inorganic phosphate. Approximately 200 mg. of P (inorganic) are found in 100 cc. of juice pressed from the skeletal muscles of the rat.[1] A potentially

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[1] Unpublished measurements made in this laboratory.

TABLE II.

Urine Nitrogen and Inorganic Phosphate Determinations.

Rat No.	Date.		Weight.	Urine P (24 hrs.).	Urine N (24 hrs.).	Ratio N:P.	Remarks.
			gm.	*mg.*	*mg.*		
68	Mar.	31	.67				Fast commenced.
	Apr.	1		Trace.	94	100+	Convulsions.
	"	2		7.0	167	24	"
	"	3		4.8	88	18	"
	"	4	51.5	4.6	107	23	"
	"	5	52	1.2	61	51	Slight convulsions.
	"	6		1.3	75	58	No convulsions.
	"	7		1.2	67	56	" "
	"	8	48.5	1.3	34	26	x-Ray taken.
	"	9		2.1	45	21	
	"	10		1.6	39	24	
	"	11	43.5	1.6	39	24	
49	Apr.	6	173				Fast commenced.
	"	7	157	0	171	100+	
	"	8	149	0	78	100+	Convulsions.
	"	9	144	1.5	54.5	36	Died.
76	May	8	108.5				Fast commenced.
	"	9		0	13	100+	Convulsions.
	"	10		12.9	150	11.6	"
	"	11		10.5	120	11.4	Slight convulsions.
	"	12	80	10	94	9.4	No convulsions.
	"	13					Died.
83 a	May	24	124				Fast commenced.
	"	25	121.5	Trace.	111.5		Convulsions.
	"	26	112.5	"	104.6		Died.
76 a	May	8	116.5				Fast commenced.
	"	9		0	84	100+	
	"	10		8.9	126	14.2	Convulsions.
	"	11		7.0	100	14.3	"
	"	12	89	6.2	99	16.0	No convulsions.
	"	13		1.6	72.5	45.0	" "
	"	14		Trace.	33.3		" "
	"	15		1	41	41	" "
	"	16		1	41	41	" "
	"	17	77	Trace.	22		Died.

TABLE II—*Concluded.*

Rat No.	Date.		Weight.	Urine P (24 hrs.).	Urine N (24 hrs.).	Ratio N:P.	Remarks.
			gm.	*mg.*	*mg.*		
83	May	24	101				Fast commenced.
	"	25	92.5	Trace.	128.6	100+	Convulsions.
	"	26	88.5	3.3	128.8	39	"
	"	27	87	5	119	24	"
	"	28	84.5	2.8	70	25	Slight convulsions.
	"	29		3.0	56.5	19 ·	No convulsions.
	"	30	85	3.0	56.5	19	" "
	"	31	85	Trace.	28	60±	" "
	June	1	82	1.2	73	61	" "
	"	2		3.0	60	20	" "
	"	3	76	3.0	60	20	" "
44	Mar.	31	96.5				Fast commenced (after
	Apr.	1	85	Trace.	73.5	100+	receiving 6 min. of cod
	"	2		13.2	66.7	5.1	liver oil). Convulsions
	"	3		12.1	72.5	6.0	began on 2nd day as
	"	4	71	3.0	20.8	6.5	usual; died on 4th day.

large source is thus indicated from which, during fasting, phosphate may be expected to enter the plasma as a result of muscle tissue destruction for energy purposes, and in amounts probably significant as regards the observed rise in plasma phosphate concentration. In order to obtain information, in roughly quantitative terms, of the extent of muscle tissue destruction and of the accompanying phosphate release, fasting rachitic rats were placed in metabolism cages and measurements of nitrogen and of inorganic phosphate were made in successive daily collections of urine. The data obtained are given in Table II.

As a means of appraising the significance of the amounts of nitrogen found in the urine in terms of phosphate released, the following roughly derived datum is offered. Assuming that the urine nitrogen is entirely from destroyed muscle tissue, 10 mg. of urine N should, according to the relative amounts of N and P in muscle protoplasm, indicate release of phosphate to an extent which would, if entirely received by the blood, raise the level there

by 10 mg. of P per 100 cc. in a rat weighing 100 gm.[2]   It is at once
evident from the urine N values in Table II that wastage of proto-
plasm occurs to an extent which should provide many times the
amount of inorganic phosphate required to produce the observed
rise in the blood plasma.   The nitrogen-phosphorus ratios in
Table II give interesting information regarding the retention of
released phosphate.   From the data given by Katz[2] the N:P
ratio in muscle protoplasm may be taken as 17.   If the N and P
in the urine, during the fasting periods, be regarded as chiefly from

TABLE III.

Blood Findings in Rats Killed after Varying Periods of Fasting.

Duration of fast.	Average weight at start of fast.*	Average weight when killed.*	Blood sugar.	Blood P (inorganic).	Comment.
		Normal rats fasted.			
hrs.	*gm.*	*gm*	*mg.*	*mg.*	
0	96 (2)	96 (2)	148	7.6	At start of fast.
22	94 (5)	85 (2)	104	5.2	No convulsions.
64		79 (1)	111	4.9	" "
140		57 (2)	78	5.2	" "
		Rachitic rats fasted.			
0	82.6 (5)	82.6 (5)	138	2.5	At start of fast.
26	83.5 (2)	72.5 (2)	74	9.8	Height of convulsions.
45	83.0 (2)	70.5 (2)	100	7.5	Active convulsion (45 hrs.)
68	75.5 (2)	60.0 (2)	167	7.7	Slight " (68 ").

* Figures in parentheses indicate the number of rats used for a deter-
mination.

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this source, a rise in this ratio is required to indicate retention of
released phosphate.   As may be seen in Table II during the
1st day of fasting, phosphate does not appear in the urine in
measurable amounts although a considerable excretion of nitrogen
occurs.   An initial retention of phosphate is thus suggested,
although it is of course probable that a part of the urine N is from

[2] Katz, J., *Arch. ges. Physiol.*, **63**, 18 (1896).   His figure for total P in
fresh muscle tissue, *viz.* 2.0 mg. per gm., is here used.   N is taken as 34 mg.
per gm. and the blood volume of a 100 gm. rat as 6 cc.

the fore period diet, and does not represent destroyed protoplasm. Direct measurements of blood phosphate, however, show, in agreement with the absence of phosphate in the urine, a large increase in plasma concentration. On the 2nd day, and for several days thereafter, phosphate and nitrogen enter the urine in amounts producing a ratio which is roughly the same or in some instances considerably above the magnitude of the N:P ratio in muscle tissue. These rough data do not warrant decision as to whether or not released phosphate is retained during this period. Following it, however, there occurs a large rise in the N:P ratio which must indicate extensive retention (see Rats 68, 76 a, and 83 in Table II). It is an obvious probability that this represents phosphate deposition in the bony tissues. If this is the case, it is interesting that deposition begins abruptly after an initial interval of several days. This high N:P ratio, it may be noted, persists for 2 or 3 days, and then, in surviving animals, falls to approximately the level preceding the rise.

In order finally to rule out hypoglycemia as a possible cause for the convulsions in our rats (a phenomenon noted by Josephs in relation to convulsions in children (6)), we measured blood P (inorganic) and blood sugar at various intervals after commencing the fast of both rachitic and normal rats, using two rats usually for each determination. Results appear in Table III. The sugar values are not essentially different, whereas interestingly the trend of change in blood phosphate is downward in fasted normal rats in direct contrast to the rise found in fasted rachitic rats.

SUMMARY.

As has been shown by Cavins, fasting of rachitic rats produces in the first 24 to 36 hours a marked elevation of the inorganic phosphate of the blood, accompanied by severe tetany. A moderate depression of calcium is also found.

As regards the source of the phosphate producing this rise, a study of the partition of phosphorus in the plasma and in the red cells of rachitic rats before and during fasting demonstrates that increase of plasma inorganic phosphate is not derived from the other phosphorus factors of the blood. Measurements, however, of nitrogen in the urine indicate a destruction of body protoplasm to an extent easily sufficient to account for the observed rise in

plasma phosphate.   Comparison of the N:P ratio found in the urine with that for muscle tissue indicates an extensive retention of phosphate released by tissue destruction, and no doubt deposited in healing bone.

Measurements of blood sugar demonstrate that the convulsions, occurring in fasting rachitic rats, cannot be referred to hypoglycemia.

Normal rats do not develop tetany when fasted.   They do not show the elevation of blood phosphate seen in rachitic animals, and their survival period is several times as long.

In conclusion, the writer wishes gratefully to acknowledge the valuable assistance afforded by Dr. James L. Gamble both during completion of the work and in criticism of the manuscript.

For the production of rickets in our experimental animals Diet 3143 of McCollum (2) was utilized.

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A NOTE ON AMMONIUM CREATININE PICRATE AND ITS POSSIBLE USE IN THE PREPARATION OF CREATININE.

By ISIDOR GREENWALD.

(*From the Harriman Research Laboratory, the Roosevelt Hospital, New York.*)

In 1924, Greenwald and Gross (1) reported that when a mixture of equivalent quantities of ammonium picrate and creatinine picrate was recrystallized several times, pure creatinine picrate was obtained. Although it was not definitely so stated, it would naturally be inferred that ammonium creatinine picrate does not exist. In more recent experiments it has been found that this compound does exist but that it readily dissociates.

As we had occasion to prepare a considerable quantity of creatinine, it seemed worth while to attempt to recover as the picrate some of the 20 per cent of the calculated yield of creatinine that is ordinarily lost with the ammoniacal filtrate (2,3). This was evaporated, *in vacuo*, to a small volume, and then treated with an excess of picric acid. The crystals that separated melted at 241°, far above the melting point of creatinine picrate. Analysis showed these to contain 77.3 per cent picric acid and 23.4 per cent nitrogen. Upon recrystallization, the picric acid content fell to 76.9, to 76.8, and then to 76.0 per cent. Further recrystallization was not attempted. The calculated values for ammonium creatinine picrate are 77.9 per cent picric acid and 23.85 per cent nitrogen.

In the three recrystallizations, approximately 12 liters of water were employed for approximately 250 gm. of picrate. If this had been an equimolecular mixture of ammonium and creatinine picrates, there would have been 105 gm. of ammonium picrate present. Since filtration was at approximately 20°, the 12 liters of mother liquid could have dissolved 122 gm. of ammonium picrate (4) (more than was present) and the product of the third

recrystallization should have been nearly pure creatinine picrate. Actually, only a slight degree of separation was obtained.

Upon dissolving equivalent quantities of creatinine picrate and ammonium picrate in hot water and allowing the solution to cool, crystals containing 75.8 per cent picric acid were obtained. Upon successive recrystallizations the picric acid content fell to 75.2 and then to 74.8 per cent, thus slowly approaching the value for creatinine picrate of 67 per cent.

Apparently, in the one experiment of 4 years ago, larger quantities of water had been used for the recrystallizations and the separation of pure creatinine picrate was more rapidly obtained.

Sa'urated solutions of the crystals containing 76.0 per cent picric acid contained 272 mg. per 100 cc. at 20° and 192 mg. at 10°. If these weights were of pure ammonium creatinine picrate they would correspond to solubilities of 0.0046 M and 0.0032 M respectively. The molar solutions are nearly the same as those of creatinine picrate at the same temperature (0.0054 M and 0.0038 M) but are far lower than those of ammonium picrate (0.042 M and 0.028 M) (4).

Apparently, ammonium creatinine picrate has a real existence but because of its ready dissociation into its components and because its molar solubility is almost exactly that of creatinine picrate though less than that of ammonium picrate, it is difficult to obtain in a pure condition.

The formation of this ammonium creatinine picrate may be used to advantage in increasing the yield of creatinine obtained from creatine. The ammoniacal filtrate is evaporated, *in vacuo*, with a bath temperature of not over 40°, to about one-half its volume. Meanwhile the amount of creatinine expected is calculated from the amount of creatine taken and the amount of creatinine already recovered. 2 equivalents of technical picric acid for each equivalent of creatinine expected are dissolved in hot water. The evaporated solution is added and the mixture is allowed to cool. The crystals are filtered, pressed out, and washed with a little water. The yield is practically quantitative.

The crystals are then ground in a mortar with concentrated hydrochloric acid (3). The mixture is filtered and washed with hydrochloric acid and then with a little ice water. The filtrate

is extracted with benzene in a continuous extraction apparatus. After all the picric acid has been removed the acid liquid is diluted and some animal charcoal, previously extracted with hydrochloric acid, is added and the mixture is boiled and then filtered. The almost colorless filtrate is evaporated on a hot-plate until crystallization begins. It is allowed to cool and is then placed in a vacuum desiccator over sodium hydroxide and calcium oxide. When nearly dry, the pasty mass is cooled to 0° and mixed with ice-cold ammonium hydroxide solution, with the use of about 0.7 cc. for each gm. of picrate originally employed. After stirring and cooling to 0°, the snow-white creatinine is filtered off, washed with ice-cold ammonium hydroxide until free from chlorides, and then with methyl alcohol. The creatinine obtained is snow-white and the yield is 60 per cent of the calculated.

Practically all the remaining creatinine can be recovered as the ammonium creatinine picrate by evaporating the ammoniacal filtrate, *in vacuo*, to one-third its volume and adding this to the press-cake of picric acid and ammonium chloride obtained as a residue in the decomposition of the ammonium creatinine picrate.

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THE MINERAL CONSTITUENTS OF CRANBERRIES.*

By FRED W. MORSE.

(From the Department of Chemistry, Massachusetts Agricultural Experiment Station, Amherst.)

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The major portion of the nation's supply of cranberries (the fruit of *Vaccinium macrocarpum*) is produced in southeastern Massachusetts, commonly called "The Cape" because Cape Cod forms the apex of its converging shores. The proximity of the ocean produces a noticeable effect on the ground waters of this region, which is shown by a high normal figure for chlorine in uncontaminated spring-waters (5). However, in supplying mineral nutrients for human consumption, cranberries from Cape Cod may fairly represent that fruit.

In the early years of this experiment station, ash analyses were made of a wide variety of vegetables and fruits, including cranberries, and a compilation of these data has been issued from time to time in its annual reports. The analyses included only the so called fertilizer constituents. Sherman (4) has included cranberries in his tables but the source of the analytical data is not given.

As part of a study of the cranberry, determinations were made of its mineral constituents.[1] The material was taken from four half barrel packages supplied by the American Cranberry Exchange, whose courtesy is gratefully acknowledged. The packages were received at intervals during the early part of 1926 and the fruit represented the crop of 1925.

Samples weighing 500 gm. each, were prepared by cutting each berry in halves, spreading the material in thin layers on trays, and

* Published by permission of the Director of the Massachusetts Agricultural Experiment Station.

[1] Credit is due Mr. Robert Wishart, temporarily employed by the Experiment Station, who performed much of the analytical work.

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drying in a large oven heated by steam. When brittle, the samples were cooled in the air, and weighed as air-dry. They were next ground in an iron mortar until all material passed through a 1 mm. mesh sieve; it was then bottled for subsequent analysis.

For the determination of the mineral constituents, charges of the air-dry material equivalent to 100 gm. of fresh fruit were used. Methods of the Association of Official Agricultural Chemists (1) were followed in the determinations of the constituents except potassium and iron. Potassium was precipitated and weighed as the perchlorate. Iron was determined in specially prepared charges of the cranberries, which had been dried without cutting

TABLE I.

Percentages of Mineral Constituents in Fresh Cranberries.

	Crop of 1925.	Early data.
Water..	88.44	
Ash...	0.158	0.18
Potassium oxide.............................	0.068	0.086
Sodium oxide................................	0.003	0.012
Calcium "	0.018	0.033
Magnesium oxide............................	0.009	0.012
Phosphorus pentoxide.......................	0.019	0.026
Sulfur.......................................	0.005	
Chlorine.....................................	0.004	
Iron..	0.00022	
Manganese...................................	0 00057	

and ground in a porcelain mortar in order to avoid contact with iron in their preparation. The iron was measured by the colorimetric thiocyanate method after incineration and solution of the material.

The results of our analyses are given in Table I together with the early analyses mentioned calculated to the basis of fresh fruit.

The iodine content of fresh cranberries has been previously reported and was 26 parts of iodine per billion, ranging up to 35 parts in berries grown nearest the sea (3).

In comparison with other fruits, cranberries have relatively little ash. A number of ash determinations have been made in the course of our cranberry studies and the range has been

from 0.15 per cent to 0.21 per cent. Therefore cranberries contain no striking amount of the common mineral constituents.

Manganese has until recently been omitted in most analyses of foods, but is now attracting more and more attention as a significant element. Lindow and Peterson have published data for manganese in a long list of foods (2). Their results on fruits show these cranberries to be comparatively high in the element.

The alkalinity of the ash of cranberries was determined by the direct titration of the ash of nine varieties of the fruit and calculation of the results in terms of cc. of normal alkali required by the ash from 100 gm. of fresh berries. The range was between 1.9 and 2.4 with an average of 2.2. Sherman calculated the alkalinity from his data to be 1.8 (4).

Summarizing, fresh cranberries generally contain less than 0.2 per cent of total ash, which has an alkalinity of about 2. The individual mineral constituents of the cranberry form very small percentages of the whole fruit.

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STUDIES ON THE METABOLISM OF LEVULOSE WITH A COLORIMETRIC METHOD FOR ITS DETERMINATION IN BLOOD AND URINE.*

By RALPH C. CORLEY.

(From the Department of Bio-Chemistry of the School of Medicine, Tulane University, New Orleans.)

The study of the metabolism of carbohydrates other than glucose has been handicapped at least in certain respects by the absence of methods for the determination of one in the presence of others. Following the entrance into the blood of a sugar, changes in the total reducing power of the blood may or may not represent the changes in concentration of the former. Isaac (1920) has emphasized certain of these points and has presented figures to show that when levulose gained entrance to the blood stream there was no parallelism between its concentration and that of the total blood sugar. Since the glucose and fructose concentrations in these results of Isaac were based on calculations from polariscopic and reduction determinations, they probably are not unobjectionable, for small variations within the limits of experimental error may make great differences in the calculated results.

Other estimations of circulating levulose have been rarely reported in the literature. There has been Seliwanoff's reaction for the detection of fructose, but this has not been very satisfactorily applied for quantitative determination. Folin and Berglund (1922) were able to obtain positive tests for levulose in blood filtrate if there were present as much as 5 mg. per 100 cc. of blood.

Kronenberger and Radt (1927) have made a series of observations on the levulose in the blood following enteral administration. A large number of their determinations were made by a method elaborated by one of them based on this resorcinol-hydrochloric

acid reaction. Later they abandoned their own method to use one published by van Creveld (1927). This employs the blue color produced when levulose is heated with diphenylamine and hydrochloric acid. The proteins were removed by precipitation with mercuric chloride, the filtrate treated with 25 per cent hydrochloric acid and an alcoholic solution of diphenylamine, and the mixture heated for 15 minutes on the water bath. After cooling and shaking with amyl alcohol to extract the color, comparison was made with standards prepared from solutions of the sugar in bichloride filtrates.

Since this method has several disadvantages including difficulty of measurement in a colorimeter,[1] it has seemed desirable to modify it to make it more convenient, and if possible to apply it to tungstic acid filtrates. In an attempt to find the most satisfactory one, a variety of procedures has been tried. Several have been reasonably practicable, but since the one finally adopted has been the most unobjectionable, the others may be disregarded.

The method that has been employed is as follows: 1 volume of the solution to be analyzed, 0.5 volume of concentrated hydrochloric acid, and 0.1 volume of a 20 per cent alcoholic solution of diphenylamine in a large test-tube are heated in a boiling water bath for 15 minutes and then cooled. It has been found convenient to close the tube with a 1-hole rubber stopper with the hole stuffed with glass wool. After being cooled, the mixture apparently may be kept indefinitely before the remainder of the determination is completed. Shaking the solution with a third volume of liquid (melted) phenol causes the immediate absorption of the diphenylamine together with the color. The addition of 0.5 volume of 95 per cent ethanol renders the mixture homogeneous and suitable for colorimetric comparison, which may be made immediately although this is not necessary. The color tends to darken slightly on standing. Standards are prepared similarly and simultaneously from solutions of levulose. It is felt preferable to make these latter at fairly frequent intervals from a 1 per cent stock solution, with use of a preservative as toluene. Since

[1] Since this paper was prepared for publication, there has come to attention a further communication of Radt (*Biochem. Z.*, **198**, 195 (1928)), in which there have been reported modifications of the original method of van Creveld, enabling a more satisfactory colorimetric measurement.

1 mg. of levulose per cc. of solution gives a fairly intense color, the standards have been made to range downwards from this concentration. Similarly if necessary the solutions to be analyzed have been diluted to within this range.

This method has been found satisfactory in the analysis of aqueous solutions, urine, and tungstic acid blood filtrates. It has been possible to make recoveries of 97 to 103 per cent of levulose added in aqueous solution. Occasionally, however, the accuracy has been somewhat less, particularly with low concentrations. Recovery of levulose added to urine has been more or less satisfactory. The results have been comparable to those with water when the urine has been greatly diluted. However, in more concentrated urines, considerable interference has been encountered, the color obtained being darker than it should be and frequently of a somewhat different shade. As yet no attempts have been made to remove interfering substances from the urine to make it possible to determine smaller amounts of levulose, that is, without great dilution.

It has been possible to recover 95 to 105 per cent of the levulose added to blood. Tungstic acid filtrates contain substances that cause the development of a faint greenish blue coloration, that increases slightly the apparent color given by levulose. This is almost certainly attributable only slightly to the presence of glucose but further than this no decision as to its origin is now possible. While theoretically dilutions and standards should probably be made from tungstic acid filtrates, as the influence is rather constant, it has been felt possible to use water, to make a small frequently determined correction, without introducing significant error, except possibly when the levulose concentration is very low. In general, the higher the concentration, the more accurate have been the results obtained.

Glucose has been found to yield about 3 per cent of the color of levulose. A few other sugars as xylose, d-arabinose, l-arabinose, and galactose have been found to cause the development of color comparable in intensity to that of glucose. This method is theoretically applicable to the determination of the levulose in any substance yielding it on acid hydrolysis. A few trials with sucrose under the same conditions gave results comparable to those with an equal mixture of glucose and levulose. Thus 1 mg. of sucrose

was equivalent to 0.533 mg. of levulose. Since the theoretical value is 0.526 mg., the contribution of glucose to the color development is somewhat lower than might be expected.

The diphenylamine employed has been obtained from the Eastman Kodak Company. An old stock of this substance available in the laboratory was found absolutely unsuitable for this determination. The reason for this is unknown. The supply may have been impure or possibly had deteriorated on standing.

A study has been made of certain aspects of the metabolism of levulose in the rabbit, to determine the concentration of this sugar in the blood and urine following administration, by use of this method. Attention is to be called to the fact that the accuracy of the values for the amounts of fructose excreted in the urine decreases as the concentration falls, by virtue of the interfering influence of certain constituents when present in more than slight concentrations. With very low renal elimination, the values recorded are to be considered merely as approximations, but nevertheless as probably indicating the actual presence of levulose in the urine. The total reducing power of the blood has been determined by the method of Shaffer and Hartmann (1920–21). Male rabbits previously fasted for periods of 24 to 48 hours have served as the subjects for these experiments. At the start, a blood sample was collected from the marginal vein of the left ear and the urine expressed from the bladder. Qualitative tests for protein and reducing sugars were made on these urines. Definite melituria has not been encountered, while one animal with a spontaneous albuminuria was discarded. In the feeding experiments, the sugar in approximately 25 per cent solution was given by stomach tube, the latter being rinsed with several cc. of water. Blood samples (2 to 3 cc.) were drawn at frequent intervals, usually about 30 minutes apart. Observations were customarily discontinued at the end of 2 hours, when levulose had nearly disappeared from the circulation in normal animals. Parenteral administration of the sugar in approximately 20 per cent solution has been made by way of the large vein of the right ear, the injection requiring from 1 to 3 minutes according to the volume. A blood sample was collected immediately, with subsequent ones at about 30 minute intervals over a period of an hour and a half, at the end of which time circulating levulose approached disappear-

ance. The urines have been taken after the last bleeding in all types of experiments. In view of the fact that a number of the animals were used repeatedly, the non-protein nitrogen of the blood has been determined to check the possibility that the continual excretion of comparatively large amounts of sugars might injure the kidney. No evidence of such an effect has been observed.

Unless otherwise indicated, levulose values are expressed as such, this sugar having practically 90 per cent of the reducing power of glucose. This accounts for the seemingly anomalous

TABLE I.
Effect of Orally Administered Levulose.

Rabbit No.................	59			54		
Weight, *kg*.................	1 60			1.45		
Ingested.						
Water, *cc*.................	40			40		
Levulose, *gm*.......	10			10		
Time of feeding........ ...	10 03 a.m.			9.47 a.m.		
Time of fasting, *hrs*........	24			42		
Urine volume, *cc*..	4			10		
	Time.	Total sugar.	Levu-lose.	Time.	Total sugar.	Levu-lose.
		mg. per cent	*mg. per cent*		*mg. per cent*	*mg. per cent*
Blood samples.	Before in-gestion.	94		Before in-gestion.	107	
	10 43 a.m.	96	10	11.02 a.m.	133	16
	11.03 "	89	10	12.02 p.m.	136	4
	11.33 "	115	6	1.35 "	146	
	12.03 p.m.	133	5			
Urine sugar, *mg*.....			10		20	13

results in a number of protocols in which the levulose value is given as exceeding that of the total sugar, which is expressed as glucose. In the tables reporting levulose as glucose, the figures merely represent 90 per cent of those obtained for the former as such. Except as stated in the discussion, each experiment reported is representative of a number of similar studies.

Table I records a number of observations made following oral administration of levulose to rabbits. The sugar appeared in the blood stream in small amounts, the concentration attaining a maximum in about an hour, with practically complete disappear-

ance in the course of the 2nd hour.   It is to be noted that the
presence of the fructose did not necessarily cause a rise in the
total blood sugar (Rabbit 59).   There has been practically com-
plete agreement among various investigators that the blood sugar
is not markedly increased when this substance is fed.   (Folin
and Berglund, 1922; Bodansky, 1923; Reinhold and Karr, 1927).
The results of Kronenberger and Radt (1927) are in general
mostly in accord with those of the present communication, in that
with the exception of two experiments there was no very marked
accumulation of levulose in the blood.

It has seemed of interest to study the concentration of levulose
in the blood after ingestion in various types of experimentally
produced liver injury.   Phosphorus, hydrazine sulfate, and chloro-
form have been employed for this purpose.   No histological studies
have been made of the hepatic tissue, but when death has super-
vened, macroscopic examination of the organ on autopsy always
showed striking gross manifestations of abnormality.   After find-
ing that about 1 mg. of phosphorus per kilo, a dosage that has been
employed by others, had no effect on the rate of removal of circu-
lating levulose, notwithstanding evident hyperglycemia, the
amounts given have been progressively increased to as much as
20 mg. per kilo, the only obvious change however being in the
enormous increases in the resulting hyperglycemias (Rabbits 64
and 65, Table II), associated with decided melituria, with however
little levulose excreted.

Hydrazine sulfate (Rabbit 64, Table II) and chloroform (Rab-
bits 72 and 61, Table III) in single doses approaching as nearly as
possible to too quickly lethal amounts, decreased the tolerance
for levulose as evidenced by total blood sugar, without increasing
the amount of circulating levulose.   Table III presents the only
experiment (Rabbit 61) in which liver injury was associated with
any evident influence on the concentration of fructose in the blood
after enteral administration.   The total blood sugar rose strikingly
and manifested no tendency to drop within the 2 hour period of
observation.   Levulose appeared in the blood in a comparatively
large amount in 30 minutes and was still present in a slightly
greater quantity even when the experiment was discontinued.   It
is to be noted that this animal received a total of 3.5 cc. of chloro-
form in the course of the preceding 8 days.   3 weeks later its levu-
lose tolerance was found to be normal.

TABLE II.

Effect of Orally Administered Levulose in Phosphorus and Hydrazine Poisoning.

Rabbit No.	61*			65†			64‡		
Weight, kg.	1.49			1.27			1.55		
Ingested.									
Water, cc.	40			40			40		
Levulose, gm.	10			10			10		
Time of feeding	10.08 a.m.			11.12 a.m.			11.21 a.m.		
Time of fasting, hrs.	24			40			24		
Urine volume, cc.	5			7			6		
	Time.	Total sugar. *mg. per cent*	Levulose. *mg. per cent*	Time.	Total sugar. *mg. per cent*	Levulose. *mg. per cent*	Time.	Total sugar. *mg. per cent*	Levulose. *mg. per cent*
Blood samples.	Before ingestion.	79		Before ingestion.	76		Before ingestion.	110	
	10.45 a.m.	123	14	11.42 a.m.	133	10	12.26 p.m.	246	23
	11.08 "	144	16	12.12 p.m.	192	12	1.28 "	201	16
	11.38 "	137	8	12.42 "	214	13	2.33 "	169	
	12.08 p.m.	120	5	1.42 "	192	10			
Urine sugar, *mg.*		6	7		150	Trace.		31	Trace.

* 4.8 mg. of phosphorus at 5.52 p.m. on preceding day.
† 24.0 mg. of phosphorus at 2.20 p.m. on preceding day.
‡ 220 mg. of hydrazine sulfate at 9.25 p.m. on preceding day.

TABLE III.

Effect of Orally Administered Levulose in Chloroform and Phlorhizin Poisoning.

Rabbit No.	72*			61†			63‡		
Weight, *kg.*	1.02			1.18			1.22		
Ingested.									
Water, *cc.*	40			40			40		
Levulose, *gm.*	10			10			10		
Time of feeding.	11.27 a.m.			10.25 a.m.			11.08 a.m.		
Time of fasting, *hrs.*	24			24			24		
Urine volume, *cc.*	3			7			7		

	Time.	Total sugar.	Levulose.	Time.	Total sugar.	Levulose.	Time.	Total sugar.	Levulose.
		mg. per cent	*mg. per cent*		*mg. per cent*	*mg. per cent*		*mg. per cent*	*mg. per cent*
Blood samples.	Before ingestion.	87		Before ingestion.	78		Before ingestion.	75	
	12.33 p.m.	180	15	10.55 a.m.	165	24	11.38 a.m.	124	6
	1.34 "	231	10	11.25 "	192	23	12.08 p.m.	106	3
				11.55 "	222	26	12.38 "	120	4
	2.43 "	243	11	12.20 p.m.	221	29	1.38 "	135	
Urine sugar, *mg.*		20			54	Trace.		459	28

* 1 cc. of chloroform at 9.15 p.m. on preceding day.

† 1 cc. of chloroform 8 days before ingestion; 1.5 cc. of chloroform 4 days before ingestion; 1 cc. of chloroform at 2.30 p.m. on preceding day.

‡ 0.5 gm. of phlorhizin at 2.15 p.m. on preceding day.

In general, it is felt that the results of the present communication are in accord with those of other authors, with regard to the effect of levulose in causing hyperglycemia both in normal animals and those with various types of liver abnormalities (Bodansky, 1923–24). However the results do not indicate a parallelism between total sugar and levulose in the blood. The explanation of the relationships involved is obscure and it is felt that the data are inadequate as yet to furnish an answer.

In the present investigation, no attempt was made to render the animals completely diabetic with phlorhizin. A single dose has

TABLE IV.
Effect of Intravenously Administered Levulose.

Rabbit No.	60		73	
Weight, *kg*	1 55		2.12	
Injected.				
Water, *cc*	10		21	
Levulose, *gm*	1.55		4.24	
Time of fasting, *hrs.*	24		24	
Urine volume, *cc.*	30		42	
	Total.	Levulose as glucose.	Total.	Levulose as glucose.
Blood sugar, *mg. per 100 cc.*				
Before injection	97		80	
After "	364	257	751	647
30 min. after injection	205	103	286	216
60 " " "	128	13	154	65
90 " " "	120		123	13
Urine sugar, *mg*	212	195	1257	1300

been administered on the day before the experiment, inducing a marked glycosuria. After feeding levulose (Rabbit 63, Table III), the total sugar rose somewhat, but the levulose curve in the blood was essentially normal.

Enteral administration unquestionably represents the physiological method of entrance to the body, but interpretation of results so obtained is rendered complicated by interreaction of various more or less known factors. Without implying that it is devoid of disadvantages of its own, intravenous administration may yield valuable information in regard to the metabolism of a substance. The amount presented to the body for disposal may be

accurately controlled, and the time rate of change followed.   The
present observation that the disposal of levulose is not appre-
ciably and consistently influenced by various conditions is suscep-
tible to more than one explanation, either that these factors are
without significance in the metabolism of the sugar, or that they
cannot decrease the ability of the tissues to handle it below that
necessary for the amounts that reach them during absorption.

TABLE V.

Effect of Intravenously Administered Levulose in Chloroform Poisoning.
Rabbit 57, 1.95 kilos; fasted 24 hours.

Time.	Blood per 100 cc.		Remarks.
	Total sugar.	Levulose as glucose	
	mg.	*mg.*	1.5 cc. chloroform at 2.10 p.m., Mar. 4.
Before injec-tion.	92		3.90 gm. levulose in 20 cc. water at 10.30 a.m., Mar. 5.
10.32 a.m.	653	581	
11.03 "	295	211	
11.30 "	223	86	
12.00 m.	170	30	25 cc. urine, 1093 mg. total sugar, 1132 mg. levulose. Fed oats. Fasted from 8.00 a.m., Mar. 6; 2 cc. chloro-form at 8.03 p.m., Mar. 6.
Before injec-tion.	107		3.70 gm. levulose in 19 cc. water at 9.23 a.m., Mar. 7.
9.27 a.m.	727	653	
9.56 "	390	328	
10.23 "	370	197	
10.53 "	331	144	15 cc. urine, 708 mg. total sugar, 837 mg. levulose.
3.00 p.m.			Died.

A study has been made of the effect of various types of con-
ditions on the rate of removal of intravenously injected levulose.
It was found early that the rapidity of removal made comparatively
large doses desirable and necessary if significant differences were
to be obtained.   0.5 gm. per kilo disappeared from the blood in
less than 30 minutes.   After twice this amount, circulating levu-
lose was practically completely removed at the end of an hour,
urinary elimination accounting for about 15 per cent (Rabbit 60,

Table IV). When 2 gm. of levulose per kilo were given a small amount remained in the blood after 90 minutes (Rabbit 73, Table IV), although approximately one-third had been excreted in the urine. No evidence has been obtained that the intravenous injection of levulose significantly influences the glucose of the blood in the normal animal, the two values having a rather constant difference. When the levulose disappeared the blood sugar returned to what may be considered as approximately normal values.

TABLE VI.

Effect of Intravenously Administered Levulose in Several Types of Intoxication.

	Total.	Levu-lose as glucose.	Total.	Levu-lose as glucose.	Total.	Levu-lose as glucose.
Rabbit No	50*		59†		64‡	
Weight, *kg*...............	2 10		1.69		1.30	
Injected.						
Water, *cc*.........................	21		17		13	
Levulose, *gm.*	4 20		3.40		2.60	
Time of fasting, *hrs*..	42		24		24	
Urine volume, *cc*....	50		40		21	
Blood sugar, *mg. per 100 cc.*						
Before injection................	110		115		63	
After "	745	667	758	682	622	464
30 min. after injection..........	392	244	336	200	250	170
60 " " "	317	89	213	79	122	34
90 " " "	273	30	180	18	118	8
Urine sugar, *mg*.................	1193	1301		1049	896	695

* 24 mg. of phosphorus at 2.00 p.m. on preceding day. Died during night.

† 225 mg. of hydrazine sulfate at 8.06 p.m. on preceding day.

‡ 0.5 gm. of phlorhizin at 5.30 p.m. on preceding day.

The results of Table V are of particular interest in that they offer the only evidence of a profound influence on the rate of disposal of circulating levulose. After the injection of 1.5 cc. of chloroform, the amount of fructose in the blood at the end of 90 minutes was somewhat but not strikingly more than has been observed in control experiments. With a repetition 2 days later with an additional administration of 2 cc. of chloroform, the blood contained 144 mg. of levulose per 100 cc. at the conclusion of the period of

observation, although the urinary elimination was of practically the same magnitude as during the first study. With milder degrees of chloroform poisoning there was a rough parallelism between total sugar and levulose in the blood, but this did not obtain under the more rigorous conditions. In the latter case although levulose was being removed from the circulation, the total blood sugar did not keep pace, the difference tending to increase.

Treatment with several mg. of phosphorus per kilo of body weight has been found to have no effect on the disposal of intravenously injected levulose, either as regards total sugar or levulose content of the blood. With increasing dosages, however, slight influences were manifested. The maximum effect was obtained with 11 mg. of phosphorus per kilo (Rabbit 50, Table VI). There remained considerable levulose in the circulation after 90 minutes, while the non-levulose sugar of the blood rose and was still at a very high level at this time.

With rabbits poisoned with hydrazine, intravenously injected levulose left the blood almost as rapidly as has been found in the normal, but there was exaggerated hyperglycemia (Rabbit 59, Table VI).

In general it may be said that treatment of rabbits with various substances considered to be destructive of hepatic tissue, has been followed by significant inhibition of the rate of disposal of intravenously injected levulose only following massive doses. Considering the likelihood that these agents affect other tissues as well, it is not felt justifiable to conclude that the results in these cases are due to a specific influence on the liver, but possibly rather to general tissue intoxication.

Phlorhizin poisoning has not been found to exert any influence on the disposal of intravenously administered fructose (Rabbit 64, Table VI).

There has been a considerable accumulation of information in regard to various aspects of the relationships of the metabolism of levulose to that of glucose. The reports of the effects of levulose on insulin intoxication have been varied (Noble and Macleod, 1923; Herring, Irvine, and Macleod, 1924; Voegtlin et al., 1924–25; Moschini, 1924). Wierzuchowski (1926) reported that insulin had no influence on the rate of removal of levulose, administered intravenously to dogs at a constant rate, as evidenced by the

total blood sugar values. On the other hand, Basch and Pollak (1927) felt that insulin increased the tolerance for intravenously injected levulose in rabbits. Cori and Cori (1927, *a*) found that insulin did not have an influence on the tolerance for intravenously injected fructose in rats, but that a surplus of insulin led to an increased oxidation of fructose (1927, *b*) and that insulin affected the manner of disposal of fructose (1928).

A great number of experiments has been carried out in regard to the relationship between insulin and rapidity with which intravenously injected levulose is removed from the circulation. While

TABLE VII.

Effect of Intravenously Administered Insulin and Levulose.

	72		56	
Rabbit No..............................	72		56	
Weight, *kg*..............................	1 04		1.95	
Injected.				
Water, *cc*..............................	13		20	
Levulose, *gm*..............................	2.08		3.90	
Insulin, *units*..............................	20		15	
Time of fasting, *hrs*..............................	38		42	
Urine volume, *cc*..........................	10		35	
	Total.	Levulose as glucose.	Total.	Levulose as glucose.
Blood sugar, *mg. per 100 cc.*				
Before injection..	88		81	
After "	533	450	769	703
30 min. after injection...........	273	178	175	108
60 " " "	87	54	104	51
90 " " "	49	4	76	15
Urine sugar, *mg*..................		340	1225	1280

the insulin has not been accurately standardized for this purpose, 2 units (recently purchased Eli Lilly preparation) per kilo invariably have been found to cause convulsions in fasting normal rabbits. Furthermore as will be reported in the near future, when equal doses of galactose have been given with 2 to 4 units of insulin per kilo, hypoglycemic shock has occurred. It is felt desirable to make these statements to answer a possible objection that certain of the results are attributable to the use of an insulin preparation of inferior potency.

Insulin injected intravenously simultaneously with the levulose

has been found to have no demonstrable effect on the rate of removal of levulose from the blood (Table VII). Insulin dosages of from 2 to 20 units per kilo of body weight have been employed without varying the results. The seeming inverse relationship between insulin dosage and urinary excretion in Table VII is not considered of real significance since a comparison with a number of other experiments does not bear out the same relationship.

In view of the reports of some investigators that levulose is not effective in protecting against insulin shock, particular attention is called to the findings that when insulin was injected with levulose in doses as great as 20 units per 2 gm. of levulose per kilo, convulsions have never been observed, the animals being kept under observation for 5 to 6 hours after the conclusion of the experiment proper. The explanation might be advanced that the insulin had been inactivated by the levulose but in the absence of direct evidence the matter must at present be left in abeyance (*cf.* du Vigneaud, 1927). In the present experiments the insulin was added to the levulose solution and the mixture allowed to stand an hour at a room temperature of 20–26°. After being kept in the incubator for 10 to 15 minutes to approximate body temperature, injection was made.

In accord with the absence of symptoms of intoxication, the blood sugar was not observed to drop to what are to be considered definitely convulsive levels, no values falling below 49 mg. per 100 cc. of blood. It is of interest to note however, that the non-levulose of the blood at times dropped to quite low levels. Thus as shown in Table VII (Rabbit 72) the difference between 87 mg. and 54 mg. represents 33 mg. of sugar other than levulose. The value of 49 mg. per cent 30 minutes later presumably represents an upward tendency of the glucose of the blood.

The results obtained with the simultaneous intravenous administration of insulin with levulose are susceptible to a variety of explanations. They may merely indicate an inactivation of the insulin, but a somewhat more logical suggestion would probably be that levulose normally leaves the blood before the insulin has begun to manifest any considerable effect. Furthermore this period of suspended action of the insulin may allow the levulose to be converted to glucose or some other substances that can oppose the action of insulin. With these factors in mind, a number

of further studies have been made, the levulose injection being preceded by the subcutaneous or intravascular administration of the insulin.

<div align="center">TABLE VIII.</div>

Effect of Intravenously Administered Levulose after Injections of Insulin.

Rabbit No.....	68		65		69		72	
Weight, *kg*......	1.25		1.15		1.25		1.05	
Injected.								
Water, *cc*..........	12 5		12.0		12.5		10	
Levulose, *gm.*	2.50*		2.30†		2.50‡		2.10§	
Time of fasting, *hrs.*	24		38		24		38	
Urine volume, *cc*.................	12		9		12		13	
	Total.	Levulose as glucose.	Total.	Levulose as glucose.	Total.	Levulose as glucose.	Total.	Levulose as glucose.
Blood sugar, *mg. per 100 cc.*								
Before injection...............	66		89		33		76	
After "	733	626	566	498	570	527	602	566
30 min. after injection.........	236	182	101	86	205	127	194	138
60 " " "	75	43	30		84	36	59	7
90 " " "	39	4	50		49	4	43	
Urine sugar, *mg*................		488		339		497	403	420

* 20 units of insulin subcutaneously 54 minutes before injection; convulsions 6 minutes after last blood sample; relieved by glucose.

† 20 units of insulin subcutaneously 76 minutes before injection; convulsions 23 minutes before last blood sample; relieved by injection of 0.5 gm. of glucose. Violent convulsions 5 hours after termination of experiment; relieved by glucose.

‡ 10 units of insulin subcutaneously 107 minutes before injection. Premonitory symptoms of intoxication 19 minutes before injection; levulose injected during mild convulsion; relief in 10 to 15 minutes with no recurrence of attacks.

§ 15 units of insulin intravenously 63 minutes before injection. Animal weak and dizzy at time of injection of levulose but soon recovered. Mild convulsion at time of last blood sample; relieved by glucose.

If the time interval was much less than 60 minutes, no obvious differences were observable (Rabbit 68, Table VIII), but when periods of an hour or longer elapsed between the injection of the insulin and levulose more definite influences on the rate of removal of circulating levulose have become evident (Rabbits 65 and 69,

Table VIII). The renal elimination was quite low. It is to be observed that in contradistinction to the results with the simultaneous insulin administration, levulose did not consistently give protection from the intoxicating effects of previous insulin treatment. Indeed there appeared to be a correlation between insulin shock and the rate of removal of circulating levulose. Furthermore levulose gave more or less permanent relief when administered during a period of convulsions.

When the insulin was given by way of the vein, its action was manifested more rapidly (Rabbit 72, Table VIII), and the removal of levulose from the circulation was accelerated.

TABLE IX.

Effect of Intravenously Administered Sucrose.

Rabbit 50, 2.00 kilos; fasted 24 hours.

Time.	Blood per 100 cc.		Remarks.
	Total reducing sugar.	Sucrose.	
	mg.	*mg.*	
Before injection.	136		2.00 gm. sucrose at 9.24 a.m.
9.26 a.m.	128	526	
10.23 "	113	157	
11.25 "	132	43	
1.10 p.m.	117		20 cc. urine, 51 mg. total reducing sugar, 1413 mg. sucrose (747 mg. levulose).

While in general the present series of experiments in regard to the effect of insulin on the disposal of levulose in the rabbit are in accord with the results of others who failed to demonstrate an influence as evidenced by urinary excretion, still it is felt that rather definite evidence has been obtained that insulin under certain conditions at any rate can affect the rate of disposal of intravenously injected levulose. The previously mentioned report of Cori and Cori (1927, *b*), that a surplus of insulin led to an increased oxidation of levulose might be advanced as one explanation of how the rate of removal of this sugar from the blood may be influenced.

Table IX exemplifies several experiments in regard to the disposal of parenterally administered sucrose. The values for the total reducing substances of the blood showed no consistent variations, while the sucrose was removed from the circulation in 3 hours or less, a large portion appearing in the urine.

SUMMARY.

1. Levulose appeared in the blood in small amounts after enteral administration to rabbits.

2. Mild poisoning with what are considered hepatotoxic agents had little influence on the levulose present in the blood after enteral administration. Under more rigorous conditions a certain effect has been observed in a few cases.

3. Following the intravenous injection of 2 gm. per kilo of body weight, levulose practically disappeared from the blood of rabbits in 90 minutes.

4. Except with massive doses, liver poisons had little influence on the rate of disposal of intravenously injected levulose in rabbits.

5. Levulose injected intravenously simultaneously with insulin has been found to protect the rabbit against the latter, without however there being any striking influence on the rate of removal of the circulating levulose.

6. If insulin has been given subcutaneously or intravenously an hour or more previous to the intravenous injection of levulose, insulin shock has been observed on numerous occasions and levulose has disappeared more rapidly from the blood.

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INTERFACIAL ADSORPTION AS A FACTOR IN THE CLOTTING OF BLOOD PLASMA.

By J. M. JOHLIN.

(*From the Marine Biological Laboratory, Woods Hole, and the Department of Biochemistry, Vanderbilt University School of Medicine, Nashville.*)

The results which are given here are those of a long series of experiments, which were made for the purpose of explaining, if possible, much of the existing confusion as to the effect, which organic solvents, such as ether and chloroform, have upon the complex phenomenon of blood clotting.

These solvents have been found to extract from various organs of the animal body a substance which retards the clotting of blood, but the solvents themselves when added to plasma have been found to accelerate clotting and to leave in the resulting serum a substance which would induce clotting in other samples of plasma. It apparently has not been possible, however, to extract from blood plasma any substance which would retard the clotting of plasma, nor has it been suggested that interfacial adsorption plays any part in the action of these solvents upon plasma. The results which are here given show that blood plasma can be induced to clot by removing from it, through interfacial adsorption one or more of the constituents, which ordinarily would retard its clotting. These results have been obtained not only at an interface such as plasma would produce with these liquids, but also at a plasma-gas interface and at the interface produced between plasma and solid adsorbing agents.

In attempting to isolate prothrombin from the oxalated plasma of cat blood by means of ether, Howell (1) caused the clotting of the plasma and considered that this treatment converted prothrombin into thrombin. Doyon (2) and also Billard (3) found that after exposing liver to the vapors of chloroform, they could obtain from the exudate by precipitation with acetic acid, a substance which when dissolved in weak alkali, retarded the coagulation of

blood.   Doyon (4) was able to extract a similar substance from various organs (spleen, kidney, liver, *etc.*).   Minot (5) from similar results which he obtained, with chloroform and ether on the oxalated plasma of various types of mammalian blood, concluded that prothrombin is not converted into thrombin by the action of these substances but that antithrombin is rendered inactive, thus allowing any free thrombin to bring about the clotting of fibrinogen.   He was unable to recover antithrombin from the ether or chloroform extracts of plasma or serum.   He concluded that the antithrombin in question was therefore not similar to that obtained by Doyon in his experiments.

Dale and Walpole (6) found that extraction of fowl plasma with chloroform as well as treatment with trypsin causes the formation of a powerful thrombin which is left in the plasma.   Since Jobling and Petersen (7) had found that chloroform and ether extraction removes an ester of an unsaturated fatty acid which is of the nature of an antitrypsin, Dale and Walpole thought that the effect of these solvents is to liberate the normal tryptic ferment of the blood by the inactivation of an antitrypsin, and that the subsequent tryptic action is responsible for thrombin formation.

Nolf (8) found that chloroform coagulated the oxalated plasma of birds, leaving a serum which was free from fibrinogen and which when added to a solution of fibrinogen would cause it to clot.   He concluded that the ability of bird plasma to keep from clotting is not due to the lack of the constituents essential to clotting, but to the fact that the quantitative relationship of the tendencies to favor and to retard coagulation is such as to favor the retardation; that chloroform does not directly destroy any anticoagulation principle but leads to the production of large amounts of thrombin (9).   He found further that fibrinogen precipitated by chloroform could be redissolved in a saline solution and then reprecipitated by thrombin (10), and that serum which has been kept in a sterile condition and has lost its power to coagulate plasma, has its ability to cause clotting restored to it by the action of chloroform (11).

Cekada (12) found that chloroform, ethyl alcohol, and carbon tetrachloride activate prothrombin to thrombin in the absence of calcium ions and that ether and methyl alcohol not only did not bring about this activation but that their continued action pre-

vented subsequent activation by calcium. No explanation for any of these phenomena could be given. He found that large amounts of chloroform precipitate fibrinogen and prothrombin from oxalated plasma, but that no substance with the characteristic of an antithrombin could be obtained. A similar but smaller precipitate was produced by ether. His conclusion is that chloroform, *etc.* act directly on prothrombin and activate it to thrombin.

Stuber, Focke, and Shen (13) have also found that inactivated serum (*i.e.*, serum in which all thrombin had apparently been converted into metathrombin) can be reactivated by extracting it with chloroform, and made even more active by this treatment than by Schmidt's (14) alkali-acid treatment for reactivating metathrombin. They found, however, that the chloroform extract, when dissolved in a sodium chloride solution and added to a thrombin solution did not retard the coagulation of fibrinogen.

Mills (15) has shown that cephalin which is known to cause the clotting of plasma can be extracted from dried goose plasma even when the plasma has been entirely freed of cells and platelets.

This diversity of opinion concerning the effects of organic solvents upon blood plasma is no doubt due to the varied conditions under which observations were made. The evidence given is not sufficient to harmonize the different points of view which are expressed.

EXPERIMENTAL.

It is well known that the blood of fish, especially of those belonging to the shark family will not clot spontaneously if it has been properly taken. The plasma can be so completely separated by centrifuging from the cells that scarcely a particle of solid material can be seen in it under a high power microscope. Such plasma can usually be kept without clotting for an indefinite period without the use of an anticoagulant, and furnishes, therefore, a convenient material for coagulation experiments. Aside from the fact that the addition of an anticoagulant would bring into consideration an additional variable, it was desirable to use plasma free from an added anticoagulant, as one of the objects of these experiments is to show the presence of such a substance naturally existing in the blood.

In the present investigation, plasma obtained from fish blood was used.   The fish were killed by pithing the brain, and, after carefully drying the peritoneal cavity with absorbent paper toweling, the blood was drawn from the dorsal aorta with a lumbar puncture needle.   It was immediately centrifuged and the clear plasma was used at once in the tests which were made.   Frequent tests showed this plasma to be free from blood cells and other solid particles.   In the course of this investigation more than 300 individual experimental tests were made with the plasma obtained from a large number of dogfish and sand sharks.

Effects of Extracting Plasma with Ether.

Ether suggests itself as the most logical of the organic solvents which might be used to extract plasma.   It is one of the best of the stable organic solvents, is not readily soluble in aqueous solutions, and the small amounts which will dissolve can later be readily removed by the mere application of a partial vacuum.   It has the further interest of being an anesthetic which is frequently admitted into the blood and tissues of the human body.

The ether which was used was Baker's pure, analyzed ether, distilled over sodium.   The immediate effect to be observed, when fish plasma is shaken with ether, is that of emulsification. If too small an amount of ether is used (1 part of ether to 1 part of plasma) it will emulsify completely to form a solid emulsion which no amount of high speed centrifuging will break up.   (Intermittent rubbing with a glass rod and centrifuging is effective in breaking it up.)   Larger amounts of ether (5 parts of ether to 1 part of plasma) produce a less firm emulsion which, upon centrifuging, readily separates into a layer of ether, a layer of treated plasma,[1] and an intervening layer composed of fibrinous matter and small amounts of ether.

If the process of shaking plasma with ether is carried on long enough (10 minutes) the treated plasma layer will, after centrifuging, have been sufficiently defibrinated to remain liquid indefinitely.   When, however, the process of shaking is carried on

[1] The term "treated plasma" is used in preference to "serum" because plasma treated with an adsorbing agent is not always so completely defibrinated that it will not clot, as will be evident in several of the experiments which will be given later on.

for a very short time (half a minute) the plasma layer will usually not be so completely defibrinated and will clot spontaneously. This clotting sometimes takes place while the mixture is being centrifuged. At other times it is possible to separate the different layers obtained by centrifuging and to remove all ether from the treated plasma layer by means of a partial vacuum before this treated plasma layer clots. That it is, therefore, not the mere presence of ether in the treated plasma which causes it to clot will be shown more definitely later on. ·

Action of Ether-Treated Plasma upon Untreated Plasma.

The treated plasma which has been shaken with ether until it is completely defibrinated and, therefore, incapable of clotting, will, when it is added to untreated plasma, almost invariably cause the latter to clot. To determine this effect the following experiment was carried out.

0.2 cc. of ether-treated plasma was evacuated to remove all possible traces of ether, and 1.0 cc. of untreated plasma was added to it. As a control experiment 1.0 cc. of the same untreated plasma, without any other additional substance, was placed in a tube of the same size.

The time required for clotting, when ether-treated plasma was added in these small amounts to untreated plasma was found to vary with individual specimens. In the case of one plasma the addition of ether-treated plasma caused it to clot in 20 seconds. In other instances the clotting time was several minutes to an hour, and occasionally several hours. Out of a large number of different plasmas thus tested, but one was found which could not be induced to clot by this procedure. In this instance, however, even the addition of cephalin was so inefficient as to produce but a loose clot after standing 48 hours. In the case of all other plasmas a firm clot would usually be obtained within a reasonably short period of time.

It was found that considerable variations in the time of shaking the plasma with ether did not produce any variations in the time required for the induction of clotting in untreated plasma by the addition of ether-treated plasma. When different samples of plasma were shaken with ether for 3, 5, and 10 minutes and the usual small portions then added to untreated plasma, the time of clotting was exactly the same in each instance.

Control experiments were necessary because occasionally, though rarely, some plasma would clot spontaneously upon standing. This was at first thought to be due to the admission of air bubbles when the blood was drawn from the fish. It was found, however, that when the most extreme care was used to exclude tissue fluids and air bubbles, spontaneous clotting might take place in the course of a few hours.

Effect of Mere Contact of Plasma with Ether.

The above experiments lead to no conclusion as to the nature of the action of the ether upon the plasma in causing it to clot. The following experiments were carried out to distinguish between the action of ether as a suitable solvent of a substance which inhibits the clotting and the action which ether might produce by merely thoroughly saturating plasma with it.

1 cc. portions of untreated plasma were placed in 10 cc. test-tubes and above the plasma in each tube was placed a cartridge of rolled filter paper which was saturated with 1 cc. of ether. The tubes were then tightly stoppered and allowed to stand.

The plasma which was used in one of these experiments was that which was found to clot in less than 20 seconds when 0.2 cc. of ether-treated plasma was added to 1 cc. This plasma when allowed to stand in a stoppered tube in the presence of ether vapors, as just described, for a period of 16 hours, showed not the slightest trace of clotting.

In the case of another plasma the presence of ether vapors caused but a very limpid gel to form in 1 hour, while the addition of 0.2 cc. of treated p'asma to a sample of this plasma caused it to clot in 8 minutes. In no case was a firm clot formed by merely allowing plasma to remain in contact with ether vapors.

Influence of Ether Extract of Plasma upon Clotting Time of Plasma.

There was, on the other hand, the possibility that the thorough shaking of plasma with ether caused the extraction of a substance which has an inhibiting action upon those constituents which bring about the formation of a clot. When subsequent to shaking plasma with ether, the ether of the centrifuged ether layer is evaporated, there is left behind a very small residue of a material which is insoluble in water. This water-insoluble residue was,

therefore, tested for its ability to retard the action which a substance like cephalin may have in inducing the formation of a clot in plasma. That cephalin will cause the clotting of plasma is well known. A number of experiments were carried out to note if the minute amounts of material extracted from plasma by ether influenced the time required for cephalin to induce clotting.

It was found that when 0.2 cc. of a 0.5 per cent solution of cephalin was added to 1 cc. of plasma, the time required for the formation of a clot was usually much less when the ether extract of 0.5 to 1 cc. of plasma was present. Data representative of these tests are given in Protocol I.

In each test 0.2 cc. of cephalin was added to 1 cc. of untreated plasma in one tube, without any additional substance. In another tube was placed 1 cc. of untreated plasma and 0.2 cc. of cephalin emulsified with the ether residue obtained by extracting 0.5 to 1 cc. of plasma. The variation in the different tests which are enumerated is the variation of individual specimens.

Protocol I.

Plasma 1.—The plasma to which the ether extract was added formed a loose clot in 29 minutes and a firm clot in 60 minutes, while three controls in which cephalin alone was added to plasma clotted firmly in 17, 18, and 19 minutes.

Plasma 2.—In one test made by adding cephalin to plasma in the presence of the ether extract a loose clot formed in 130 minutes and a firm clot in 170 minutes. In a duplicate test no clot had formed in 2 hours but clotting took place when the test stood overnight, while duplicate controls in which cephalin alone was added to plasma both clotted in 75 minutes.

Plasma 3.—The sample containing the ether extract in addition to the cephalin did not clot in 30 hours, while the control in which cephalin alone was added to plasma clotted in 7 hours.

The clot formed by the addition of cephalin in the presence of the ether extract of plasma not only required a longer time, but was less firm than the clot formed when the extract was absent. In its properties, it was not unlike the clot formed by the addition of cephalin to plasma in the presence of small amounts of heparin. In the three series of experiments of Protocol I there is to be noted a

pronounced difference in the time of clotting when cephalin was added to plasma without any additional substance and when it was added in the presence of the minute amounts of the ether extract of plasma. In many other cases the difference in clotting time was not so pronounced. Whatever difference there was to be observed was always such as to indicate that the ether extract had a retarding influence upon the action of clotting.

Experiments with Chloroform.

Since chloroform had previously been known to remove proteins from solution by surface adsorption, and to induce clotting in blood plasma, no difficulty was anticipated in obtaining results with chloroform similar to those which were obtained with ether. A slight difference in the action of this substance from that of ether should, however, be noted. Chloroform is emulsified with plasma much more readily than ether, and plasma which has been treated with chloroform too vigorously will induce clotting in untreated plasma less readily than when treatment is less vigorous.

Protocol II.

Plasma 1.—1 cc. of plasma was shaken with 5 cc. of $CHCl_3$ for 3 minutes; then centrifuged and placed under a partial vacuum. 0.2 cc. added to 1 cc. of untreated plasma induced clotting in 5 hours. The control remained liquid.

Plasma 2.—1 cc. of plasma was shaken with 5 cc. of $CHCl_3$ for 3 minutes; then centrifuged and placed under a partial vacuum. 0.2 cc. added to 1 cc. of untreated plasma induced clotting in $2\frac{1}{2}$ hours. The control remained liquid.

Plasma 3.—(a) Three different 1 cc. portions were shaken with 5 cc. portions of $CHCl_3$ for 1, 3, and 5 minutes; each portion centrifuged, placed under a partial vacuum and 0.2 cc. of each added to a 1 cc. portion of untreated plasma. The plasma treated for 1 minute induced the untreated plasma to clot in $2\frac{1}{2}$ hours, that treated 3 minutes induced clotting in $3\frac{1}{2}$ hours, that treated 5 minutes induced clotting in untreated plasma in $3\frac{3}{4}$ hours. A control remained liquid.

(b) 1 cc. of plasma was shaken with 5 cc. of $CHCl_3$ 3 minutes, then centrifuged, and the plasma layer shaken with a second 5 cc.

portion of CHCl₃ 3 minutes, again centrifuged, and the plasma layer shaken with a third 5 cc. portion of CHCl₃ for 3 minutes, again centrifuged, placed under a partial vacuum to free from CHCl₃ and 0.2 cc. added to 1 cc. of untreated plasma. The latter was liquid at the end of 3 hours and did not clot until 7½ hours. The control remained liquid.

Plasma 4.—Repetition of experiment Plasma 3 (*b*). The plasma treated 3 minutes induced clotting in 85 minutes. The plasma treated three successive times for 3 minutes with different portions of CHCl₃ induced clotting in 4 hours. A control remained liquid.

Plasma 5.—1 cc. of plasma was shaken with 5 cc. of CHCl₃ for 3 minutes. 0.2 cc. added to 1 cc. of untreated plasma induced clotting in 1 hour.

The data of Protocol II show that when plasma is shaken with chloroform for 1 minute, it induces clotting more readily that when this treatment is carried on for 3 or 5 minutes. These data also show that when plasma is shaken with chloroform but 3 minutes and then centrifuged, evacuated, and added to untreated plasma, it will cause the latter to clot more readily than when the process of shaking and centrifuging is carried out three successive times, with three different 5 cc. portions of chloroform, before it is evacuated and added to the untreated plasma.

In Protocol II 1 cc. portions of plasma were always emulsified with 5 cc. portions of chloroform and then centrifuged. When this treatment was repeated with fresh portions of chloroform, these successive treatments were found to remove increasingly smaller amounts of precipitated matter from the plasma. The chloroform-treated plasma was always subjected to a partial vacuum until all odor of CHCl₃ had disappeared before it was added to the untreated plasma. 0.2 cc. portions of the treated plasma were added to 1 cc. portions of untreated plasma. Control experiments were always made. In these 1 cc. of untreated plasma was allowed to remain in a tube of similar size to that in which the other tests were carried out.

Effect of Emulsifying Plasma with Air.

Although the ether extract of plasma was found to retard the clotting of untreated plasma when cephalin was added to the

latter, the solvent action of ether in possibly removing an anti-prothrombin cannot alone be held responsible for the fact that ether-treated plasma causes untreated plasma to clot. This action of ether seems to be not so much that of a solvent as of a substance inducing the surface adsorption of an antiprothrombin, and setting free in the plasma an active prothrombin. The emulsification of plasma with a gas as well as with a solid produces the same effect as when it is emulsified with a liquid such as ether or chloroform. This action of interfacial adsorption at an air-plasma interface helps to explain the formation of the thin surface clots which are found to have formed when plasma has stood for some time.

In Protocol III are given repeated experiments in which 1 cc. portions of plasma were shaken with air, the resulting foam periodically removed, and the shaking continued until all of the plasma had formed a foam. This foam was allowed to stand for 15 to 20 minutes and was then centrifuged. By centrifuging, most of the plasma was again collected as a clear liquid, but a small part always separated as a fibrous, spongy clot. The air-treated plasma would sometimes clot after centrifuging, and at other times would remain liquid indefinitely.

Protocol III.

Plasma 1.—1 cc. of plasma was shaken with air until completely emulsified, and allowed to stand 15 to 20 minutes. After centrifuging, 0.2 cc. of air-treated plasma was added to 1 cc. of untreated plasma. The latter clotted in 10 hours, while a control sample remained liquid.

Plasma 2.—Upon following the above procedure, air-treated plasma caused untreated plasma to clot in 4 hours, while a control remained liquid.

Plasma 3.—Upon following the above procedure, air-treated plasma caused untreated plasma to clot in 8 hours, while a control remained liquid.

Plasma 4.—Upon following the above procedure, air-treated plasma caused untreated plasma to clot in 5 hours, while a control remained liquid.

When 0.2 cc. of air-treated plasma was added to untreated plasma the latter would clot. Control experiments, in which

plasma was merely allowed to stand in tubes of the same size as those in which the tests were made always remained liquid.

Negative Effect of Emulsifying Plasma with Paraffin Oil.

It is a well known fact that blood clots more readily when contained in a clean glass vessel than it does when the vessel is lined with paraffin or paraffin oil. Paraffin oil and plasma are quite easily emulsified. Such an emulsion will, however, in the course of a few hours completely separate into its two constituents when allowed to stand. This separation is more readily brought about by centrifuging. Such an emulsion, even though it has been violently shaken for half an hour readily separates into two layers when centrifuged, leaving at the interface barely a trace of anything resembling adsorbed fibrous material. Plasma which has been treated with mineral oil will itself not clot because of such treatment nor will it cause untreated plasma to clot when added to it. This lack of adsorption at a plasma-oil interface is of course responsible for the protective action of the oil in preventing or prolonging the time of clotting of blood plasma.

Action of Barium Sulfate and of Kaolin.

Bordet and Delange (16) found that contact of plasma with barium sulfate or other precipitates, deprives the plasma of its coagulability. Since their results were, however, obtained with oxalated mammalian plasma, it would be difficult to draw any comparison between their data and those which follow.

A paste was made of 1 cc. of plasma and 1 gm. of precipitated barium sulfate and allowed to stand, with occasional shaking, for 5 minutes. It was then centrifuged. The greater part (60 to 70 per cent) of the plasma remained absorbed by the barium sulfate. The supernatant liquid left by centrifuging, in some cases, clotted upon subsequent standing, and in others, remained liquid indefinitely. In every case, when 0.2 cc. of this treated plasma was added to 1 cc. of untreated plasma, clotting occurred. The data of Protocol IV illustrate this phenomenon. Each plasma is that of a different specimen.

Protocol IV.

Plasma 1.—The plasma treated with $BaSO_4$ remained liquid, but added to untreated plasma caused it to clot in 85 minutes.

Plasma 2.—The plasma treated with $BaSO_4$ remained liquid, but, added to untreated plasma caused it to clot in 75 minutes.

Duplicate Experiment.—The plasma treated with $BaSO_4$ clotted while centrifuged; the clot was broken up by shaking and was centrifuged again; the clear liquid then added to untreated plasma caused it to clot in 95 minutes.

Plasma 3.—The plasma treated with $BaSO_4$ clotted after standing for some time. Before it had clotted, 0.2 cc. was added to untreated plasma which caused the latter to clot in 50 minutes.

Plasma 4.—The plasma treated with $BaSO_4$ remained liquid; but added to untreated plasma, caused it to clot in 60 minutes.

Plasma 5.—The plasma treated with $BaSO_4$ remained liquid; but added to untreated plasma caused it to clot in 340 minutes. In each case, a control tube of untreated plasma allowed to stand during the period of the experiment remained liquid.

When plasma was treated with varying amounts of kaolin in a similar manner, the results obtained were far more irregular. It was found that the plasma that had been treated with kaolin would usually clot after centrifuging. If, however, while still liquid, it was added to untreated plasma, it would not always induce clotting in the untreated plasma. In a number of cases, however, clotting was induced by the addition of kaolin-treated plasma to untreated plasma, as shown by the data of Protocol V. The ratio of plasma to kaolin varies in the different instances given. In each case, however, 0.2 cc. of the supernatant liquid obtained from treated plasma was added to 1 cc. of untreated plasma.

Protocol V.

Plasma 1.—1 gm. of kaolin to 3 cc. of plasma was used in treatment; the supernatant liquid induced clotting in untreated plasma in 70 minutes. A control tube of untreated plasma remained liquid.

Duplicate Experiment.—Clotting was induced in 70 minutes. The control remained liquid.

Plasma 2.—1 gm. of kaolin to 4 cc. of plasma was used in treatment. Clotting was induced in 60 minutes. The control remained liquid.

Duplicate Experiment.—Clotting was induced in 60 minutes. The control remained liquid.

Plasma 3.—1 gm. of kaolin to 8 cc. of plasma was used in treatment. Clotting was induced in 205 minutes. The control remained liquid.

Duplicate Experiment.—1 gm. of kaolin to 4 cc. of plasma was used in treatment. Clotting was induced in 205 minutes. The control remained liquid.

It is evident from these experiments that contact with barium sulfate of kaolin does not deprive fish plasma of its ability to clot, but rather causes it to clot. The fact that kaolin-treated plasma induced clotting in untreated plasma in but a limited number of cases must be ascribed to individual variations in the plasma of different fish, just as there were variations in the time of clotting when ether-treated plasma was added to untreated plasma.

Action of Powdered Glass upon Plasma.

Nolf (17) found that precipitated calcium fluoride, calcium phosphate, and glass, ground exceedingly fine, and emulsified with plasma, cause it to clot, but he gave no explanation for the phenomenon.

That glass in contact with plasma causes the latter to clot may be observed by allowing plasma to stand in a capillary tube, as in attempts to measure the surface tension of plasma by the capillary rise method. The plasma in the capillary clots while the main bulk of the plasma remains liquid. The relatively large ratio of glass surface to bulk of liquid in the capillary must be held responsible for the clotting which takes place in the capillary.

In the following experiments both Pyrex and soft glass were used. The glass was powdered in a porcelain mortar and that between 70 and 140 mesh was used. 1 gm. of powdered glass was allowed to remain in contact with 1 cc. of plasma, with occasional shaking, for 5 minutes, and was then centrifuged. Sometimes clotting would set in before the mixture was centrifuged. At other times clotting would set in during centrifuging, or immediately after. Shaking, and additional centrifuging, would break up these clots and leave a small amount of clear supernatant liquid. As in the case of barium sulfate and kaolin

about 70 per cent of the plasma remained absorbed by the glass. Although this treatment with glass would almost invariably cause the plasma to clot, the glass-treated plasma only occasionally induced clotting in untreated plasma when added to it.   In many cases where glass-treated plasma would not induce untreated plasma to clot, it would however cause the precipitation of fibrous particles, while the control tubes, containing untreated plasma only, would remain clear.

In one instance where plasma treated with Pyrex glass was added to untreated plasma, clotting was induced in 2 minutes. In one instance where plasma treated with soft glass was added to untreated plasma, clotting was induced in 11 minutes, and in another instance in 13 minutes.

DISCUSSION.

The fact that no two plasmas behave exactly alike makes it rather difficult to standardize these experiments.   The different types of adsorbing interfaces showed a varied tendency to adsorb the fibrinogen from solution.   What other proteins are removed, and in what degree, could not, without too great complications, be determined in this investigation.   In the experiments in which ether was used to create an interface with plasma, it was definitely shown that, in addition to any fibrinogen that was adsorbed, a substance of the nature of an antiprothrombin was also removed, thus allowing a prothrombin in the plasma to bring about the clotting of the plasma.   The fact that in all other instances with the exception of that of paraffin oil, clotting was also induced by the contact of the plasma with an interface, makes it reasonable to infer that in these instances clotting was due to the surface adsorption of an antiprothrombin.   This point of view appears in harmony with Howell's explanation of blood clotting (18).

SUMMARY AND CONCLUSIONS.

Clotting experiments which were carried out with the plasma of dogfish and sand sharks showed that clotting can be induced by the contact of the plasma with substances which produce an adsorbing interface between the plasma and these substances. Such induced clotting was found to take place where an interface

had been formed between plasma on the one hand, and air, ether, chloroform, barium sulfate, kaolin, powdered soft glass, and powdered Pyrex glass on the other. No appreciable adsorption was found to take place at the interface between plasma and paraffin oil, and in these instances clotting could not be induced by the emulsification of plasma with paraffin oil. Following the emulsification of ether with plasma, the ether was found to contain a substance which retarded the clotting of plasma by cephalin. The different types of interfaces are not equally effective as adsorbing surfaces nor does the plasma of different individuals of the same species behave in a like manner. The action described in these experiments appears to be primarily that of the interfacial adsorption of a substance of the nature of an antiprothrombin, the removal of which leaves free in the plasma a substance which induces clotting.

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THE CHEMICAL STUDY OF BACTERIA.

XXIV. A PROXIMATE CHEMICAL ANALYSIS OF THE TIMOTHY BACILLUS.

By ROBERT D. COGHILL AND ORSON D. BIRD.

(From the Medical Research Laboratories of Parke, Davis and Company, Detroit.)

INTRODUCTION.

During the past few years a comprehensive chemical and biological investigation of the tubercle bacillus has been conducted in different research centers[1] under the auspices of the Research Committee of the National Tuberculosis Association. The chemical investigation of the bacterial cells has been conducted largely at the Sterling Chemistry Laboratory of Yale University. To date, the research has been confined almost entirely to one strain of the organism, the H37 (human). The research program (1) was organized, however, with the idea of extending the work to other acid-fast bacteria, such as the avian and bovine tubercle bacilli, *Bacillus lepræ*, and the timothy bacillus. Through the courtesy of Parke, Davis and Company, it has been possible this past summer to conduct the preliminary investigation of the timothy hay bacillus, *Mycobacterium phlei* (Moeller), in their laboratory, in accordance with the outlined program.

This organism, as the name indicates, is found associated with grass, hay, grains, *etc*. It is very closely related to the tubercle bacillus, being an acid-fast, Gram-positive rod, with the general appearance and growth characteristics of the tubercle bacillus. It is, however, entirely non-pathogenic. There were two primary objectives to be attained in this investigation. The first was to

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[1] Cornell University Medical College, Hygienic Laboratory, Washington, D. C., Rockefeller Institute, Russell Sage Foundation, Sprague Institute, Yale University, H. K. Mulford Company, Parke, Davis and Company.

see what distinctive differences could be found, chemically, between two organisms which were so closely related biologically, yet differed so widely in their pathogenicity. The second was to obtain, as nearly pure as possible, samples of the carbohydrates, proteins, fats, and the nucleic acid of the cell for comparative biological tests. Sabin and Doan (2) have noted characteristic effects produced in healthy and tuberculous animals by various fractions from the human strain of the tubercle bacillus. Would the corresponding fractions from a non-pathogenic organism produce similar effects? It was hoped that this preliminary investigation would give results suggesting a significant lead for future study in this field.

The bacteria used were grown entirely on Long's synthetic medium (3) made from chemicals conforming to the standards of purity accepted by the u.s.p. In this way it was assured that any carbohydrates, proteins, and fats found would be bacterial products, as the medium used contained none of these. The growth of bacilli was carried on in 1 liter Pyrex bottles, 200 cc. of medium in each bottle, and at a temperature of 37°. The age of the bacteria ranged from 28 to 31 days. The heavy pellicle was filtered off on Buchner funnels, washed once with distilled water, and dried at 40° in a vacuum. This left an easily pulverized product of a characteristic orange color, which contained 8.0 per cent of nitrogen and 1.6 per cent of phosphorus.

<div align="center">EXPERIMENTAL.</div>

A. Fats.—It was evident from the first few experiments that the fat content of the timothy bacillus was less than that of the H37. However, in an experiment performed upon the residue left after ether and dilute sodium hydroxide extractions, it was noticed that an acid hydrolysis set free considerably more fat. Accordingly, the following experiment was performed.

17.2 gm. of bacteria were extracted in a Soxhlet extractor with ether for 18 hours. At the end of that time the ether had ceased to remove any more material. Upon evaporation of the ether, 0.322 gm. of fat were obtained, this representing a 1.87 per cent yield. The colorless residue was now subjected to a similar treatment, with chloroform. This yielded 0.146 gm. of fat, or 0.85 per cent.

Thus the total fat extractable by the above mentioned solvents was only 2.72 per cent.

The residue was now warmed on a water bath for 2 hours with 100 cc. of 20 per cent hydrochloric acid. A voluminous black precipitate was formed. This was filtered off, thoroughly washed with water, and allowed to dry in the air. It was then extracted with cold chloroform. After removing the solvent from the chloroform extract and subsequent drying of the resulting fat in a vacuum desiccator for 2 days, 2.569 gm. of fat were obtained. This represents a 14.93 per cent yield. The total fat content then amounts to 17.65 per cent.

B Proteins and Nitrogen Distribution.—Two experiments were performed to determine nitrogen distribution, the same method being used in each case.

100 gm. of the desiccated bacteria were defatted by four successive extractions with 1 liter portions of ether. The bacteria were shaken during the day with the solvent, allowed to settle overnight, and the supernatant liquid decanted the next morning.

The cell residue, after being air-dried, was ground in a ball mill with 1 liter of water, a little chloroform being added as a preservative. Two 4 hour periods of grinding were given on successive days, the bacteria being kept in the ice box between times. After the second grinding, the macerated mass was centrifuged, giving a water extract and a residue which was given a second water extraction in precisely the same manner that the first one had been made. The first and second aqueous extracts were combined, and the volume noted. It was then forced through a Mandler candle, giving a sparkling, clear solution. An aliquot of this was analyzed for nitrogen, the results being corrected for losses incurred during the filtering process.

An originally water-soluble protein was obtained from this extract by the addition of enough glacial acetic acid to make the final concentration of 3 per cent, determined as optimal by a preliminary experiment. The precipitated protein was allowed to settle, the supernatant liquid decanted, and the residue centrifuged. The protein was washed in the centrifuge once with 3 per cent acetic acid, twice with alcohol, twice with ether, and finally dried in a vacuum desiccator. The filtrate from the protein was used to prepare the water-soluble carbohydrate to be described later.

To the residual mass from the water extraction was added enough dilute sodium hydroxide to make a liter of 0.5 per cent alkali. It was then ground in the ball mill for 2 hours, centrifuged, and the residue returned to the ball mill for another 2 hour period of grinding with 700 cc. of 0.5 per cent alkali. While the second alkaline extraction was being made, the first extract was acidified with acetic acid in order to prevent further action of the

TABLE I.

Nitrogen Distribution in Timothy Bacillus.

Cell fractions.	Weight.		Nitrogen.		Total N in bacteria.			
	Experiment I.	Experiment II.	Experiment I.	Experiment II.	Experiment I.		Experiment II.	
	gm.	*gm.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Ether-soluble.............	0.6				None.			
Water-soluble............					14.5		11.9	
Protein.................	1.72	2.08	14.65	15.12		3.2		3.95
Non-protein............						11.5		7.95
Alkali-soluble............					16.4		15.15	
Protein.................	3.4	3.09	14.8	14.97		6.3		5.8
Non-protein............						10.1		9.35
Alcohol-soluble after above treatment..............					1.9		0.65	
Final residue.............	51.0	50.0	9.35	10.05	59.9		63.1	
Total.................					92.7		90.8	

alkali on the protein. This destructive action was evident from the odor of ammonia in the ball mill.

The second alkaline extract was added to the first and the combined solution made just alkaline to litmus. It was then filtered through a Mandler candle. The resulting clear extract was analyzed for nitrogen and the figure corrected for losses in the Mandler candle, the volume of extract having been noted before and after filtration. The alkali-soluble protein was then precipitated by the addition of glacial acetic acid to a concentration of 3 per cent, and washed and dried as described above in the case of the water-soluble protein.

The residue from the alkaline extraction was washed once with 3 per cent acetic acid and three times with 95 per cent ethyl alcohol, the washings being filtered and analyzed for nitrogen. After the alcohol washings, ether was used until no further pigment was extracted, and the white residue dried in a vacuum desiccator.

In Table I is recorded the amount of each product obtained, its nitrogen content, and the approximate nitrogen distribution in the bacteria. The figures represent results obtained in two separate experiments. All figures are corrected for measured losses in manipulation.

The nitrogen unaccounted for is probably lost as ammonia during the alkaline extraction.

C. Carbohydrate Fraction.—It is best, for purposes of economy of material, to obtain the water-soluble carbohydrates from the filtrate from the water-soluble protein. It can also be obtained by the same procedure directly from the water extract of the bacteria, but in this case the protein is lost.

The filtrate from the water-soluble protein, obtained as described above, is treated with a solution of lead acetate until no further precipitate is formed. The precipitate is filtered off, and the filtrate evaporated at 40° in a vacuum. As soon as it has been reduced to a thick syrup, it is treated with basic lead acetate and enough ammonia added to make the solution decidedly alkaline.

The lead precipitate is allowed to stand overnight, when it is washed thoroughly with distilled water, then decomposed with hydrogen sulfide. The filtrate from the PbS is evaporated in a vacuum at 40° to a thick syrup, which is further concentrated in a vacuum desiccator over sulfuric acid. When nearly dry it is thoroughly triturated with absolute alcohol, and finally washed with ether.

The carbohydrate fraction thus obtained contains reducing sugars and will give an osazone which, as yet, is unidentified. It is yellow in color and hygroscopic. This latter property is probably due, in part at least, to the presence of ammonium acetate, which is hard to remove. The yields on two preparations were 7.0 and 6.7 gm., the products containing 3.46 and 3.72 per cent of nitrogen respectively. In a third experiment, where the carbohydrate was redissolved in water and reprecipitated with the basic lead acetate, the yield was only 3.3 gm., but the nitrogen content had dropped to 1.67 per cent.

The purification and identification of this carbohydrate will be the subject of future work.

DISCUSSION.

The most important result obtained from the above experimental work is that it has been shown that there are enough gross chemical differences between the timothy bacillus and the H37 (human) strain of the tubercle bacillus to differentiate between the two organisms in the chemical laboratory.

The first point of difference noted was in the medium which was filtered from the bacteria. This medium is filtered through a Mandler candle, giving a clear solution. In the case of the H37

TABLE II.

Protein and Nitrogen Distribution in the Timothy and Tubercle Bacillus.

	Per cent of total N.	
	Tubercle bacillus.	Timothy bacillus.
Water-soluble N........	35.7	13.2
" protein N......................	0.7	3.5
Alkali-soluble N..............................	44.7	15.8
" protein N.....................	33.2	6.0

strain, addition of acetic acid to this solution gives a copious precipitate of protein, which Long and Seibert have shown to act as a potent tuberculin (4). In the case of the timothy bacillus, addition of acetic acid to the medium does not so much as produce an opalescence, the solution remaining perfectly clear. This is due to the difference in the proteins present, rather than to the lack of protein in the latter case, because saturation of the medium with ammonium sulfate produces a heavy precipitate of protein in both cases.

The next point of difference noted was in the fat content. With the same method of extraction on the H37 tubercle bacillus, which had been grown on the same medium and treated subsequently in the same manner, an average of 20 per cent of fat was obtained (5), while in the case of the timothy bacillus a complete extraction with ether in a Soxhlet apparatus gave only 1.87 per cent. Further-

more, after an alcohol-ether extraction, Anderson (6) was able to extract 11 per cent more fat with chloroform, while the timothy bacillus yielded with chloroform only 0.85 per cent.   In this connection it is interesting to note that the timothy bacillus eventually yielded 14.9 per cent of a chloroform-soluble fat, but only after hydrolysis.   In the experiment outlined above the hydrolysis was made with 20 per cent HCl, but the fat was also set free by a short treatment with 0.5 per cent sodium hydroxide when the bacteria were extracted for protein.   We have no knowledge regarding the manner of linkage of this fat fraction.

The nitrogen distribution also differs notably from that of the human tubercle bacillus.   By practically the same procedure as that described here, the tubercle bacillus yields 36 per cent of its total nitrogen to the water extract (5), while two experiments with the timothy bacillus gave 11.9 and 14.5 per cent respectively. While it is hard to get good checks on an experiment of this type, the difference here noted is far outside of any experimental error. It is also of interest to note that in the case of the tubercle bacillus only 2 per cent of the nitrogen in the water extract was protein nitrogen, while with the timothy bacillus the protein contained 27 per cent of the water-soluble nitrogen.

There are also marked differences in the amounts of nitrogen extracted by 0.5 per cent NaOH.   In the tubercle bacillus this amounts to about 45 per cent of the total nitrogen, in the timothy bacillus only about 16 per cent.   Here, however, we find the amount of protein obtained reversed.   The tubercle bacillus gives a 20 per cent yield of a protein which contains 33 per cent of all the nitrogen in the bacteria.   The timothy bacillus only yields slightly over 3 per cent of protein, which contains only 6 per cent of the nitrogen.

These differences are best illustrated in Table II.   The figures given are averages of two determinations.

SUMMARY.

1. It has been demonstrated that there are enough differences between the H37 human strain of the tubercle bacillus and the timothy bacillus to differentiate between them chemically.

2. Various preparations from the timothy bacillus are now

available to compare with the corresponding fractions of the tubercle bacillus. The results from these comparisons may indicate which fraction or fractions of the bacteria are important from the biological and therapeutic view-point.

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THE INFLUENCE OF EPINEPHRINE AND INSULIN UPON THE DISTRIBUTION OF GLYCOGEN.

By N. R. BLATHERWICK AND MELVILLE SAHYUN.

(From the Chemical Laboratory of the Potter Metabolic Clinic, Santa Barbara Cottage Hospital, Santa Barbara.)

It has been the general belief that the injection of epinephrine into fasting animals causes a depletion of the glycogen reserve in the liver. This has been one of the methods used for bringing about a glycogen deficit. A recent paper by Cori and Cori (1) submits evidence that epinephrine does not cause a disappearance of liver glycogen but, on the other hand, it brings about an increased content of the liver glycogen. This increase is believed to occur by a transfer of muscle glycogen to liver glycogen with lactic acid as an intermediary product. These authors give an excellent résumé of the literature. The purpose of our paper is to record some experiments which support the findings of Cori and Cori. In our experiments rabbits were used, whereas rats were the experimental animals of the investigation just mentioned.

EXPERIMENTAL.

The rabbits used in these experiments were all females which had previously been utilized for the assaying of insulin. They were fed a low carbohydrate diet consisting of alfalfa hay and barley once a week. During the 3 months when they were not used for insulin standardization just preceding these experiments, a little barley was added to the daily food. A fasting period of 24 hours came before the actual experiments. Blood samples, in all instances, were obtained prior to sacrificing the animals. The removal, weighing, and mincing of the liver did not require more than 2 minutes. Another person, simultaneously, prepared the muscle for the determination of glycogen. 50 gm. of tissue were taken for this estimation. Pflueger's method (slightly modi-

fied) was used. Blood sugar was determined according to the method of Folin and Wu.

One Epinephrine Injection.—Rabbits 5, 6, and 11 were each given in the forenoon 1 mg. of epinephrine subcutaneously. Another 24 hour fast followed before the blood samples were obtained, and the animals were then sacrificed. The findings are recorded in Table I.

Two Epinephrine Injections.—Rabbits 19, 45, and 61 were given 1 mg. of epinephrine in the forenoon and 4 hours later a like

TABLE I.

Glycogen Content 24 Hours after Injection of 1 Mg. of Epinephrine.

Date.	Rabbit No.	Weight.	Blood sugar.	Liver glycogen.	Muscle glycogen.
1928		*kg.*	*per cent*	*per cent*	*per cent*
Aug. 7	5	2.40	0 083	0.143	0 041
" 7	6	2.40	0.105	0 210	0 065
" 7	11	2 60	0.118	0.195	0.036

TABLE II.

Glycogen Content 24 Hours after Injection of 2 Mg. of Epinephrine in Two Doses 4 Hours Apart.

Date.	Rabbit No.	Weight.	Blood sugar.	Liver glycogen.	Muscle glycogen.
1928		*kg.*	*per cent*	*per cent*	*per cent*
Aug. 16	19	3.40	0.095	2.65	0.011
July 17	45*	2.50	0.095	3.10	0.055
" 27	61	3.25		5.10	0.010

* This rabbit was splanchnectomized.

amount. The animals were kept fasting until the next day, when they were killed. The data are presented in Table II.

Two Epinephrine Injections Followed by Insulin.—After a 24 hour fast, Rabbits 17, 18, and 20 each received 1 mg. of epinephrine in the morning, followed by a similar amount 4 hours later. They were then fasted until the 2nd day when each was given a subcutaneous injection of 1 unit of insulin per kilo. 2 hours later the rabbits were killed. Blood sugar was determined before the insulin was given and prior to death. The results are given in Table III.

One Epinephrine and One Insulin Injection.—Rabbits 8, 10, and 12 after fasting 24 hours were each given 1 mg. of epinephrine in the forenoon and 3.5 hours later 1 unit of insulin per kilo subcutaneously. They were then kept fasting until the next day when the data presented in Table IV were obtained.

Glucose Ingestion.—For comparative purposes, Rabbit 21, after a 48 hour fast, was given 1.75 gm. of glucose per kilo by stomach

TABLE III.

Glycogen Content 24 Hours after Injection of 2 Mg. of Epinephrine in Two Doses Followed by Injection of 1 Unit of Insulin.

Date.	Rabbit No.	Weight.	Blood sugar.		Liver glycogen.	Muscle glycogen.
			Initial.	After 2 hrs.		
1928		*kg.*	*per cent*	*per cent*	*per cent*	*per cent*
Aug. 10	17	2.75	0.111	0.050	3.33	0.053
" 10	18	3.00	0 095	0.065	3.25	0 047
" 10	20	3 30	0.087	0.040	2.50	0 047

TABLE IV.

Glycogen Content 24 Hours after Injection of 1 Mg. of Epinephrine Followed by 1 Unit of Insulin.

Date.	Rabbit No.	Weight.	Blood sugar.	Liver glycogen.	Muscle glycogen.
1928		*kg.*	*per cent*	*per cent*	*per cent*
Aug. 14	8	3.20	0.103	2.5	0.105
" 14	10	3.40	0.087	0.70	0.061
" 14	12	3 15	0.100	1.78	0.018

tube. 4 hours later it was killed. The blood sugar was 0.11 per cent and the liver and muscle glycogen were 3.05 and 0.09 per cent respectively.

DISCUSSION.

The data recorded in Table I, when one injection of epinephrine was administered, do not show any accumulation of glycogen in the liver. Blatherwick *et al.* (2) found an average content of 0.19 per cent of liver glycogen and 0.14 per cent of muscle glycogen in rabbits fed a similar diet and fasted for 24 hours. The limited data do not allow final judgment but it appears likely that there was an appreciable decrease in muscle glycogen.

The results, presented in Table II, show a very marked increase in liver glycogen the day following the injection of 2 mg. of epinephrine in equal doses. The muscle glycogen at this time was low; in two cases there was practically none.

The findings presented in Tables III and IV, when insulin was given following the injection of 1 and 2 mg. of epinephrine, show high values for liver glycogen and that glycogen was accumulating in the muscles. Insulin following the injection of 1 mg. of epinephrine apparently caused the deposition of glycogen in the liver, as may be seen by comparing Tables I and IV. Insulin given after the injection of 2 mg. of epinephrine appears to have changed but little the distribution of glycogen, as may be seen from Tables II and III.

The data, as a whole, support the contention of Cori and Cori that epinephrine causes glycogen to be deposited in the liver with a simultaneous decrease in the content of muscle glycogen. It is evident that not only the amount of epinephrine injected but also the time allowed to elapse before the tissues are taken for analysis have a great bearing upon the results. Further work on this problem was desirable, but since both of the authors are no longer connected with this laboratory, it was decided to publish the results already obtained.

In a recent publication by us (3), we showed that injections of epinephrine caused rabbits to become more sensitive to insulin. As an explanation of this result, we offered the suggestion that epinephrine decreased the liver glycogen and thereby rendered the animals less resistant to insulin. It is plainly evident from the data here presented that such an explanation has no basis in fact. The increased susceptibility to insulin following the injection of epinephrine is not due to a depletion of the glycogen reserve in the liver. The correct explanation awaits solution.

SUMMARY.

Epinephrine, when injected into rabbits, causes glycogen to be deposited in the liver. The muscle glycogen appears to decrease at the same time. The amount of epinephrine used and the time chosen for the experiment undoubtedly have a great bearing upon the result.

The diminished resistance of rabbits to insulin following the injection of epinephrine cannot be attributed to a depletion of liver glycogen

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THE AMIDE NITROGEN OF BLOOD.*

II. A QUANTITATIVE METHOD.

By SIDNEY BLISS.

*(From the Department of Biochemistry, McGill University, Montreal,
Canada.)*

The persistent search for new methods for the estimation of the
ammonia content of blood bears adequate testimony to the unsatis-
factory nature of the ones we have.   With alterations in the tech-
nique for the estimation of the amount of ammonia in blood have
come changes in our ideas as to the absolute amounts involved in
the metabolic processes, and the changing view-point has had an
important influence on the theoretical deductions to be drawn from
experimental work in this field.

With rare exception, the available methods for the estimation of
the ammonia of blood depend upon the assumption that the am-
monia is present in blood in a form analogous to that of an ammo-
nium salt of an acid, because the ammonia is measured by aerating
or distilling from an alkaline fluid.

Parnas and Heller (1) have described an alternative method to the
vacuum distillation method.   By allowing blood to stand for 24 hours they
allow for the development of ammonia from a precursor which is named
an "ammonia mother substance," and the absolute values obtained in this
way range from 2 to 4 mg. per 100 cc. of blood.   Parnas and Klisiecki (2)
were unable to establish that such values bore any relationship to the
condition of acid-base balance, and in the latest article that has come to
the writer's attention, this method has been abandoned by Parnas, Mozol-
owski, and Lewinski (3), who therein attempt to assign physiological sig-
nificance to the very low absolute values obtained in the old standard way;
that is, by direct vacuum distillation of blood.

It has been our experience that the ammonia content of blood
rises on standing, but, contrary to the findings of Parnas and his
coworkers, we find that the maximum values are often attained

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* The first article of this series appeared in *Science* (7).

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only after a period considerably longer than 48 hours.   From what is to follow, it will be clear that the highest values obtained by Parnas and the low values obtained by him and all other workers in the field are perhaps accidental in character, and can be of no significance in the sense that an ammonia value for blood is an indication of the state of acid-base balance or measures ammonia that is of use to the body for the purpose of acid neutralization.

The excessively low values for the ammonia of blood lead one to suspect that ammonia which is formed for acid neutralization in the tissues may be masked in its transit through the blood to the kidney for excretion.   Such toxic substances as thymol and camphor are known to be conjugated with one of the normal constituents of blood, and are then carried by the blood in the form of an inocuous compound, and, analogously, the toxic nature of the ammonium ion suggests the plausibility of its linkage with some other blood constituent until it reaches the kidney.

The behavior of compounds of glucose and ammonia is suggestive.   The writer has prepared crystalline compounds of glucose and ammonia by a modification of the method described by Stone (4) and others, and found that when this substance is subjected to the ordinary aeration method for determining the ammonia content of blood only 1 per cent, or less, of the nitrogen is obtained as ammonia.   However, a few minutes contact with 0.1 N acid is sufficient to free the ammonia, and aerations of samples so treated yield 100 per cent of their nitrogen as ammonia by the direct aeration method as used for blood.

On the assumption that ammonia is transported in the blood in the form of a complex that might yield ammonia again under physiological conditions, a search was made to see whether an enzyme might be found in kidney tissue capable of liberating ammonia from a compound in blood that did not yield the ammonia by ordinary direct aeration of the blood made alkaline with sodium carbonate.   The finding of Van Slyke (5) that the addition of excessive carbonate to blood stops the usual increases of ammonia observed in blood that is allowed to stand, was confirmed by us and lent credence to the idea of an enzymatic process being involved. Such an enzyme was found in kidney tissue, and its purification and concentration has been studied in detail and will be made the subject of a separate communication.

The action of the newly found enzyme brought to light certain facts: (1) The absolute value for the "ammonia complex" of blood is many times that of the old low ammonia values. (2) The values so obtained show excellent correspondence with the physiological state (the expected variations with double nephrectomy, alkaline tide, bicarbonate feeding, *etc.*). (3) The "ammonia complex" of blood is completely precipitated with the proteins by the common protein precipitants. (4) The enzyme resembles a deamidase, probably a protein deamidase. (5) The absolute values obtained by acid hydrolysis of the protein fraction of blood are of the same order of magnitude as those obtained by enzymatic hydrolysis.

Due to the more rapid, complete, and satisfactory hydrolysis of amides by acids, as compared with enzymes, together with the labor involved in the purification of this new protein deamidase, the method for the determination of the amide nitrogen of blood was developed as follows.

Outline of Method.

After the tungstic acid precipitation of the proteins of blood, the precipitate is washed with tungstic acid to remove traces of urea, dissolved, and a portion of it subjected to hydrolysis with sulfuric acid at the temperature of the boiling water bath. After the neutralization of the sulfuric acid, the mixture is aerated with an excess of sodium hydroxide, and the ammonia Nesslerized as in the standard micro aeration method of Folin and Macallum (6).

Procedure.

Introduce 1 cc. of blood into a graduated centrifuge tube which is known to be correct at the 10 cc. mark, add 7 cc. of water, 1 cc. of $\frac{2}{3}$ N sulfuric acid, and finally 1 cc. of 10 per cent sodium tungstate. The material may be mixed with a stirring rod and the material adhering to the rod washed into the tube with water because the final volume at this point does not influence the accuracy of the method. Centrifuge and discard the liquid which can be poured off cleanly[1] from the tightly packed precipitate in the bot-

[1] A slight film of precipitated protein is partly lost in this decantation. The material so poured off was collected from thirty-six determinations, and analysis showed a loss of something less than 0.1 per cent.

tom of the tube.   The precipitate[2] is next suspended in a dilute
tungstic acid solution (made by adding 1 cc. of $\frac{2}{3}$ N sulfuric acid,
1 cc. of 10 per cent sodium tungstate, and 98 cc. of water); the
final volume at this point is also immaterial.   After being centri-
fuged for the second time, the fluid is again discarded.   To the
precipitate in the centrifuge tube next add 1 cc. of water.   With
the aid of a stirring rod the precipitate is worked up into a smooth
emulsion.   Add an additional 2 cc. of water and stir.   Next add 1
cc. of 0.9 N sodium hydroxide solution (dilute 30 cc. of 3 N sodium
hydroxide to 100 cc. to prepare 0.9 N solution) and complete solu-
tion of the precipitate is obtained almost instantaneously.   By the
addition of water the volume is now made up to 10 cc. in the centri-
fuge tube, a drop of caprylic alcohol being added to produce a
sharp meniscus, and the contents of the tube mixed by inverting it
several times.   The solution thus obtained is so nearly neutral that
it is alkaline to alizarin and acid to phenolphthalein.   2 cc. of this
dissolved protein are pipetted into a standard 25×200 mm.
Pyrex tube, and 1 cc. of a 12 N sulfuric acid solution added, giving a
final concentration of 4 N acid.   The acid, so added, is completely
mixed with the protein solution already in the tube by very gentle
agitation so as to cause the whole of the material to assume a semi-
solid condition without the material being splashed over the sides of
the tube.   The tube is covered with a watch-glass and clamped up-
right in a water bath kept at a gentle boil.   At the end of 90
minutes the tube is cooled under the cold water tap and set up for
aeration according to the standard micro aeration method of
Folin and Macallum (6).   The tube containing the sample is
fitted with a 2-hole rubber stopper; through one hole the inlet tube,
which admits the compressed air, extends to the bottom of the test-
tube; the outlet tube occupies the other hole in the stopper, and
extends on the one side to the bottom edge of the rubber stopper,
while it, in turn, conducts the air (which has been through the
sample) into an acid collection tube similar in size to the tube con-
taining the sample to be aerated.   The collection tube contains
2 cc. of 0.1 N acid.

When set up for aeration, the inlet tube that admits the com-

[2] The protein-free filtrate yields, on hydrolysis with acids, only the
amount of ammonia that is to be expected from its urea content, and is,
hence, free of measurable amounts of amide nitrogen.

pressed air (into the Pyrex tube containing the sample) is disconnected, and into it are run first 4 drops of caprylic alcohol, and then 6 cc. of 3 N sodium hydroxide. The inlet tube is again connected with the compressed air, and the air current started. The time of aeration is best determined by the time required to make quantitative recovery of ammonia in preliminary determinations with known amounts of ammonia. Under our conditions, 30 minutes are adequate. The rate of aeration is carefully controlled at the beginning, when the air is admitted very slowly. The rate is increased gradually through the 30 minutes that follow. When caprylic alcohol is used as an antifoam reagent, it remains in the acid collection tube in sufficient amount to interfere with Nesslerization, and must next be blown off by conducting the air current directly into it for nearly 5 minutes.

The amount of ammonia obtained represents that in 0.2 cc. of blood. It is washed from the acid collection tube into a 50 cc. volumetric flask with enough water to make a volume of about 40 cc. 5 cc. quantities of Nessler solution are added simultaneously both to this flask and to a standard flask containing 0.3 mg. of ammonia nitrogen. Both flasks are made up to the mark with water, and after 15 minutes standing are compared in the colorimeter. 20 divided by the reading, times 150 = mg. of amide nitrogen per 100 cc. of blood.

Accuracy of Method.

The accuracy of the method depends largely upon whether one can transfer ammonia quantitatively from one tube to another by aeration, and secondly, upon the accuracy with which one can match the two fields in a colorimeter.

Quantitative transfer of ammonia by aeration is a procedure that requires patient trial to perfect, but it is true that ammonia can be so recovered with an accuracy that is within the limits of colorimetric measurement.

There is difference of opinion about the possible accuracy of colorimetric comparisons—and the personal factor is very important. If one is careful to establish and maintain the conditions that are necessary for accurate colorimetry, it is possible to read a standard color against itself with an error of less than 1 per cent. The results shown in Table I have been obtained with the color

TABLE I.
Triplicate Estimations of Amide Nitrogen Content of Blood.

Sample No.	Blood amide N.	Average of triplicates.	Deviation from mean value.
	mg. per 100 cc.	*mg. per 100 cc.*	*per cent*
1	144.2		0.00
	143.6		0.41
	144.9	144.2	0.48
2	143.6		0.28
	144.2		0.14
	144.2	144.0	0.14
3	147.0		0.00
	146.3		0.47
	147.8	147.0	0.54
4	145.7		0.27
	146.3		0.14
	146.3	146.1	0.14
5	141.5		0.78
	140.3		0.08
	139.5	140.4	0.64
6	138.3		0.65
	137.0		0.30
	137.0	137.4	0.30
Average...			0.32

TABLE II.

Amide N of Normal Human Blood (All Samples from Cubital Vein).
All the subjects were male.

Subject.	Age.	Blood amide N.	
	yrs.	*mg. per 100 cc.*	
A. H.	23	141	1.5 hrs. after grapefruit, toast, poached eggs, coffee.
D. McI.	23	137	1 hr. after bread, eggs, tea.
G. P.	32	139	4 hrs. after Corn Flakes, tea, milk.
E. G.	16	138	3 hrs. after toast, tea.
S. N.	22	144	3.5 hrs. after toast, coffee.
U. S.	23	143	2 hrs. after fish, potatoes, bread, milk, cheese.
S. B.	35	134	1 hr. after coffee, cookies, banana.
"	35	137	3 hrs. after fish, potatoes, toast, coffee, pie.

produced by the Nesslerization of ammonia. They indicate that estimations of the amide nitrogen content of blood may be carried through in triplicate with variations of from 0.00 to 0.80 per cent from the average of the three values.

The author feels justified, therefore, in claiming that under his conditions, variations greater than 1 per cent are outside the limits of error of the method.

The values given in Table II have been obtained from normal human blood. These values supersede the slightly lower ones reported earlier (7). The difference is attendant upon further refinement of the method.

SUMMARY.

1. A method is described for the quantitative estimation of the amide nitrogen of blood.

2. The normal level of amide nitrogen for blood drawn from the cubital vein in the human varies from 134 to 144 mg. of amide nitrogen per 100 cc. of blood.

3. The unavoidable error of the method is less than 1 per cent.

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THE AMIDE NITROGEN OF BLOOD.

III. MUSCULAR EXERCISE: THE RÔLE OF AMMONIA IN THE NEUTRALIZATION OF LACTIC ACID.

By SIDNEY BLISS.

(From the Department of Biochemistry, McGill University, Montreal, Canada)

Certain chemical changes are known to occur in muscle during, and immediately following, short periods of exercise. With respect to the alteration in the acid-base equilibrium under such conditions, some facts are definitely established.

It has been shown by Barr and Himwich (1) that within a few minutes after exercise very considerable amounts of plasma bicarbonate are neutralized by acid, and the finding of simultaneous increases in blood lactic acid of more than 500 per cent leaves no doubt that lactic acid as it issues from the muscles where it was formed, combines with base, and thus accounts for the lowering of the alkaline reserve as measured in blood.

Liljestrand and Wilson (2) have shown that at such times there is a marked increase in the urinary excretion of lactic acid, although the amounts so lost to the body constitute but a small fraction of all the lactic acid which must be disposed of within a short period following muscular exercise. Unquestionably, resynthesis of the lactic acid precursor occurs, and this disposition of a considerable quantity of lactic acid is a rapid affair.

The high concentrations of lactic acid found in blood shortly after exercise are of short duration, and the return to a normal level of blood lactic acid is the net result of the simultaneous accumulation and removal of lactic acid. The two processes proceed at different rates and in different places. Lactic acid formed in the muscles of the leg is disposed of in resting muscles elsewhere in the body (3). The whole cycle of events may take from 10 to 50 minutes after short periods of muscular exercise (1).

Lactic acid which appears in such large amounts during muscular exercise disappears again with similar rapidity, and the base that was combined with the lactic acid is again available.   This phenomenon is well illustrated in the results of Barr and Himwich (3).   They found that when *leg* muscles were exercised, the CO_2-combining capacity of venous blood from the *arm* was always higher than in arterial blood because the resting muscle of the arm received blood which contained lactic acid combined with bicarbonate, and the removal of the lactic acid by the resting muscle left the blood issuing from it richer in available base.   More recently (4), the liver has been shown to play an important part in the removal of lactic acid.

It is clear, then, that the disposition of lactic acid during the recovery period involves a massive liberation of bicarbonate quite similar in amount to the initial diminution of that substance when it served for lactic acid neutralization.   Barr and Himwich observed that at no time was there an alkalosis.   This is consistent with the view that at the time of the massive liberation of bicarbonate which had been combined with lactic acid, the body was in a state of acidosis, and the net result is a return to normal.

Because we have satisfactory methods of determining plasma bicarbonate, the rôle played by fixed base in the neutralization of lactic acid appears capable of estimation.   Fixed base, however, cannot accomplish all of the neutralization that occurs.   The following quotation is Hill's (5) statement of the problem:

"The nature of this neutralisation is a very interesting and important matter. As Meyerhof has shown, the total amount of alkaline salts (bicarbonate and phosphate) present in a frog's muscle is inadequate to account for the large amount of acid known to be neutralised in severe fatigue. Moreover the rise of hydrogen ion concentration and the evolution of CO_2, if neutralisation by bicarbonate were the case, would be greater than are actually observed. It is necessary to suppose that some more effective buffer, something analogous to the haemoglobin of blood, is present in muscle, and strong evidence exists for the hypothesis of a sodium (or potassium) compound of protein, capable of neutralising acid according to the scheme:

$$Na^+ + P^- \;+\; H^+ + L^- \longrightarrow Na^+ + L^- \;+\; HP$$
(sodium protein)   (lactic acid)   (sodium lactate)   (protein)
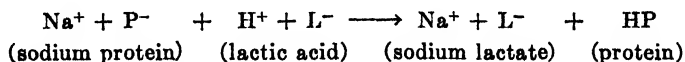
This protein is a weaker acid than H_2CO_3 or NaH_2PO_4 and it acts as the first line of defence against a rise of hydrogen ion concentration.   The

substance in question has not yet been isolated from muscle: the evidence for its existence is indirect: it is impossible, however, to explain the facts if we deny its presence."

In the past it has seemed impossible to consider that ammonia might be the missing alkali. Since the work of Folin and Denis (6) it has been the generally accepted view that blood contains only the slightest traces of ammonia, if, indeed, there be any at all. A further objection to the supposition that ammonia plays a part in the neutralization of lactic acid in muscle has been the general acceptance of the view stated by Nash and Benedict (7) that all ammonia used for acid neutralization purposes is formed in the kidney. If the neutralization of an acid by ammonia can occur only in the kidney, then one cannot ascribe any part of the neutralization of lactic acid in muscle to ammonia.

With the discovery of a deamidase in kidney tissue (8) the possibility presents itself that urinary ammonia may be formed from amides of blood proteins. If such an explanation be the true one, one must decide whether the amide nitrogen of blood plays the rôle of an ammonia precursor from which the kidney alone is able to form ammonia for acid neutralization, or whether, as has been stated by Bliss (9), ammonia formation for acid neutralization is a general tissue phenomenon. The evidence for the latter view will be considered in an analysis of the values obtained for the amide nitrogen of blood following short periods of muscular exercise.

So far as the writer is aware, the presence of ammonia in tissues has never been seriously questioned. A number of workers have reported tissue analyses which show large quantities of ammonia in various organs (10, 11), and even though recent developments of the subject suggest the need for a careful confirmation of these results, it is perhaps safe to assume that ammonia does exist in tissues.

On theoretical grounds it is reasonable to believe that when an acid appears in a tissue its neutralization will be accomplished by the different bases present in amounts that are determined by their relative strengths and concentrations. When lactic acid is formed in muscle, its neutralization must be accomplished mainly by bicarbonate, phosphate, and protein. A weak alkali like ammonia

must be of lesser quantitative importance, although there is reason
to believe that it would combine with some of the lactic acid.

Attempts have been made to demonstrate changes in the ammonia con-
tent of venous blood after exercise (12, 13), but the quantities involved are
far below the physiological range which would suffice for acid neutraliza-
tion purposes. In fact, all analyses for ammonia in blood must be called
into question as far as demonstrating that the quantities found actually
existed in a preformed condition.

We know that enzymes whose place of action is outside the vessels do
escape into the blood in small amounts, and there is every reason to believe
that "ammonia" values for blood as they now stand in the literature repre-
sent the development of ammonia during the course of the analysis, however
short and that in circulating blood there is absolutely no ammonia. An
exception to this statement must be made with regard to the ammonia
formed by putrefaction in the large intestine—for it is probable that here,
and here only, ammonia does really find its way into circulating blood,
though it cannot play an important rôle in acid neutralization, owing to
its rapid removal from blood in the liver.

Folin (14) early called attention to the possibility that the low values
established for the ammonia content of blood may represent merely post-
mortem decompositions. He said: "Neither in nephritis nor in diabetes
accompanied by pronounced acidosis do we find more than utterly insig-
nificant traces of ammonia in the blood. . . . . I am sometimes skeptical
as to whether there really is any ammonia at all in blood, whether the
traces we do find represent anything but postmortem decompositions.

"There may be something radically wrong about all methods for the
determination of ammonia in blood. Unsuspected chemical combinations
may come into play by virtue of which the ammonia is kept in merely '
potential form and is set free by the kidneys in connection with the secre-
tion of the acid urine. The ease with which ammonia combines with
certain aldehydes suggests such a possibility. This problem awaits
solution."

<div align="center">EXPERIMENTAL.</div>

<div align="center">*Significance of Amide Nitrogen of Blood.*</div>

Heretofore the amide nitrogen of the blood proteins has received
almost no attention. In former communications (15, 8), it was
pointed out that an enzyme obtained from kidney tissue is capable
of liberating from the proteins of whole blood an amount of am-
monia that is quantitatively comparable to the amount liberated
from blood proteins by short periods of acid hydrolysis, known to
be specific for amide hydrolysis.

It was inferred that the amide nitrogen of the protein fraction of whole blood might be of metabolic significance as an index of the ammonia metabolism. The following experiments furnish the evidence for such a view.

Amide Synthesis by Tissues. Intravenous Injection of Ammonium Salts.

A. Normal Unanesthetized Dogs.—The capacity of tissues to synthesize amides from ammonia, if demonstrable, offers a means of explaining not only the presence of amide nitrogen in blood, but the fact, already reported (15, 8), that the values for amide nitrogen of blood vary in the direction that would be expected if the amount of amide nitrogen in blood is related to ammonia metabolism.

In the attempt to demonstrate that tissues possess the power of synthesizing amides from ammonia, ammonium carbonate solutions were injected in different ways. The toxicity of ammonia when injected intravenously is well known.

Intravenous Injection.—A dog weighing 9.4 kilos (with femoral vessels exposed under local ethyl chloride anesthesia) received *via* the femoral vein 8.5 cc. of a 12 per cent ammonium carbonate solution. The amount injected contained 249.9 mg. of ammonia nitrogen (26.6 mg. per kilo). The typical picture of ammonia intoxication was produced. Convulsions appeared at once and made further injection impossible. During the following 10 minutes the animal lost control of the external sphincters, and vomiting was frequent. At the end of 4 minutes there was temporary respiratory standstill accompanied by generalized extensor rigidity. This was followed by a period of rapid breathing (respiration 90) which persisted, with gradually diminishing intensity, for an hour. The dog regained control of the hind limbs by the end of the 3rd hour, and made a complete recovery.

Arterial Injection.—On the following day the same dog received an exactly similar quantity of ammonium carbonate solution injected at the same rate, this time into the femoral *artery*. Absolutely no noteworthy effect followed injection into the artery. This result, duplicated repeatedly in previously unused dogs, points to the conclusion that ammonia which is injected into the blood going to the muscles of the leg is disposed of in some way

which prevents its appearance in the venous blood, and thence in the circulation generally, in amounts capable of causing symptoms of toxicity.

It is worthy of special note that the appearance of symptoms of ammonia poisoning depends wholly upon the vessel into which the ammonia is injected.   An amount of ammonia that will produce symptoms of ammonia poisoning when the injection is made into the femoral *vein* can be injected into the femoral *artery* without manifesting any disturbing symptoms whatever.   The order of injection is immaterial.   If ammonia is injected into the femoral artery of a previously unused dog no convulsions ensue.   When, on the following day, a similar amount of ammonia is injected into the femoral vein of the same dog, ammonia poisoning results.   If the order be reversed on a fresh dog, ammonia poisoning occurs with the first injection into the vein, and the subsequent injection into the artery is uneventful.

However, it is possible to inject an amount of ammonia into the femoral artery that is in excess of what the tissues of the leg can handle immediately, and in such a case, some of the ammonia escapes into the venous circulation in amounts capable of producing poisoning.

The amount of ammonium carbonate solution that can be injected into the femoral artery without producing symptoms of poisoning is in the neighborhood of 24 mg. of ammonia nitrogen per kilo of body weight injected in 12 per cent ammonium carbonate solution at the rate of about 2 cc. per minute.   Calculations on the basis of the weight of the animal are not strictly comparable in animals of widely varying size.   In animals weighing 7 kilos it is usually possible to inject 24 mg. of ammonia nitrogen (as 12 per cent ammonium carbonate solution) per kilo into the femoral artery without danger of poisoning.   It is not necessary to inject this much, however, because an injection of 20 mg. of ammonia nitrogen per kilo will produce symptoms when put into the vein, and is quite safe when injected into the artery.   Intraarterial injections of ammonia are slightly irritating, and we always remove any ammonia adhering to the outside of the needle used.   Any such irritant effect is easily distinguished from symptoms of poisoning.

Since muscular tissue can dispose of injected ammonia in such a

way as to prevent the appearance of symptoms that accompany ammonia intoxication, it becomes of interest to know whether amide synthesis can account for this protection.

The synthesis of amides from ammonia by tissues has been shown in the following way. Normal dogs have been used. They are gently secured and the femoral vessels on one side exposed under local ethyl chloride anesthesia. Ammonium carbonate solutions containing 30 mg. of nitrogen per cc. have been injected into the femoral artery at a rate that enables the tissues to handle most of the injected ammonia. The rate of injection is important, because, as stated elsewhere, too rapid injection into the artery allows considerable amounts of ammonia to escape into the venous blood without being subjected to the action of the tissues of the leg, and in such cases one gets general systemic effects rather than the local changes which it is the purpose to demonstrate here.

At short periods following the intraarterial injection of ammonia, simultaneous arterial and venous samples are taken for analysis. In this way it has been possible to study the amide content of blood entering and leaving the leg of the dog both before and immediately following the injection of ammonia. The results are shown in Tables I and II.

In establishing the significance of the amide nitrogen of blood it has seemed to us to be a point of such cardinal importance that it be shown beyond doubt that tissues do synthesize amides from ammonia that we have matched every experiment on ammonia injection with a control experiment. In the controls we have injected a solution of similar percentage concentration of salts, of the same pH, and have injected at the same rate. In some cases the control experiment has been performed upon the same animal.

It may be said at the outset that after every injection of ammonia into the femoral artery of the dog we have observed a rise in the amide nitrogen content of the blood from the femoral vein. Not only are the values for the blood of the femoral vein higher in absolute amount after the injection of ammonia, but the differences between simultaneous arterial and venous samples are always greater, in favor of the femoral vein (as high as 30 times the average experimental error for the method, and 10 times the highest observed experimental error). Also, in every control

experiment in which we have used solutions that were identical with the ammonia solutions except for the substitution of sodium for the ammonium ion, there has been complete uniformity in the results; namely, the differences between arterial and venous values

<div align="center">TABLE I.</div>

<div align="center">*Amide Synthesis from Injected Ammonia.*</div>

12 per cent ammonium carbonate (30 mg. of N per cc.) injected into the femoral artery (20 mg. of N per kilo).

The dogs were in a fasting condition.

Time after ammonium carbonate injection.	Blood amide N			Time after ammonium carbonate injection.	Blood amide N.		
	Femoral.		Venous — arterial		Femoral.		Venous — arterial
	Artery	Vein.			Artery	Vein.	
Dog 38, female; age 1 yr., wt 7.0 kilos.				Dog 37, male; age 1 yr , wt. 6.5 kilos.			
min.	mg. per 100 cc.	mg. per 100 cc.	mg.	min.	mg. per 100 cc.	mg. per 100 cc.	mg.
Preliminary.	155	147	−8	Preliminary.	144	147	+3
4 7 cc. in 4½ min.				4 4 cc. in 4 min.			
1½	152	166	+14	⅓	149	163	+14
3	156	159	+3	2⅓	150	157	+7
5	156	154	−2	4½	153	156	+3
7	156	155	−1	6⅓	152	155	+3
10	157	151	−6	9⅓	145	149	+4
Dog 32, female; age 1 yr , wt. 4.2 kilos.				Dog 31, female; age 1 yr., wt. 5.9 kilos.			
Preliminary.	110	108	−2	Preliminary.	152	152	0
3 cc. in 1½ min.				4 cc. in 3 min.			
½	113	121	+8	1	148	154	+6
2½	113	118	+5	3	150	152	+2
4½	115	118	+3	5	147	150	+3
6½	113	116	+3	7	150	152	+2
8½	110	116	+6	9	152	150	−2

have remained what they were in the samples preliminary to injection, or have fallen in value, and there is no increased output of amide nitrogen from the tissues of the leg.

The samples of blood taken after ammonia injection were ex-

amined to see whether the higher values for amide nitrogen in the vein could be traced to changes in blood concentration, and no differences of any consequence could be detected.   This was done

TABLE II.
Control Injection; No Amide Synthesis.

12 per cent sodium carbonate solution brought to same pH as for 12 per cent ammonium carbonate solution by passing CO_2 through the solution; injected into femoral artery.

The dogs were in a fasting condition.   •

Time after sodium carbonate-bicarbonate injection.	Blood amide N			Time after sodium carbonate-bicarbonate injection.	Blood amide N.		
	Femoral.		Venous – arterial.		Femoral.		Venous – arterial.
	Artery	Vein			Artery.	Vein.	
Dog 34, female; age 1 yr., wt. 6.0 kilos.				**Dog 32, female; age 1 yr., wt. 4.2 kilos.**			
min.	mg. per 100 cc	mg per 100 cc	mg.	min.	mg. per 100 cc.	mg. per 100 cc.	mg.
Preliminary. 4 cc. in 3 min.	124	129	+5	Preliminary. 3 cc. in 1½ min.	110	111	+1
⅜	123	129	+6	⅜	111	111	0
2	126	131	+5	2½	110	108	−2
4	126	129	+3	4½	108	112	+4
7	126	128	+2	7½	108	110	+2
9	124	127	+3				
Dog 35, male; age 1 yr., wt. 6.5 kilos.				**Dog 37, male; age 1 yr., wt. 6.5 kilos.**			
Preliminary. 4 4 cc. in 3 min.	125	128	+3	Preliminary. 4.4 cc. in 4 min.	131	134	+3
½	122	126	+4	1	138	126	−12
2½	122	126	+4	3½	136	136	0
4½	122	126	+4	5¾	138	140	+2
7½	121	124	+3	6¾	136	141	+5
9½	121	126	+5	10	138	141	+3

as follows: Introduce 0.5 cc. of blood into a 100 cc. volumetric flask partly filled with distilled water.   When hemolysis is complete, add 1 cc. of 1:5 dilution of concentrated hydrochloric acid to the flask.   Make up to the mark and mix.   With one sample

as the standard, any other sample may be compared with it by comparing the resulting colors in the colorimeter.

Blood concentration has been followed by total nitrogen determinations as well.   Certain changes in blood concentration have been indicated in this way, and the changes are such as to yield corrected values for the amide nitrogen of blood that are even more striking in character than those reported in Tables I and II.

Exercise Experiments.

The general procedure for an experiment has been as follows: Our dogs are fed with raw beef heart once daily at 5 p.m.   The experiments were usually started at 10 a.m.; in any case the dog had fasted for a period of some 18 hours before an experiment. Quiet dogs were selected in order that the preliminary values would represent the resting condition as nearly as possible   The dog is first secured on the table, and the femoral artery and vein exposed under ethyl chloride local anesthesia.   Before the day of the experiment the dog is put through the procedure of being tied down, and if the strangeness of the situation causes any struggling, this is repeated until the animal's confidence has been obtained; otherwise the dog is not used.

For convenience of manipulation, loose ligatures are laid around each femoral vessel.   Blood is drawn from the artery and vein simultaneously by two persons, and is received in syringes containing potassium oxalate.   The amount drawn is slightly over 1 cc.   After pads have been held on the vessels for a matter of 3 minutes, the dog is released from the table and made to run on the level as fast as he will go for exactly 2 minutes.   Counting from the end of the 2 minute period of running, simultaneous arterial and venous samples are taken at the end of 3, 10, 20, 40, and 60 minutes, the dog being kept quiet during this time.

Tables III to VIII show the results obtained in a study of the effect of exercise on the concentration of amide nitrogen in blood. The results are in harmony with those obtained by other investigators who used short periods of muscular exercise, particularly in the length of time required for the cycle of changes to take place. The changes in blood amide nitrogen are such that the return to a normal value is usually accomplished in from 40 to 60 minutes.

It is perhaps simpler to interpret arterial values than venous ones, because an individual venous sample is not necessarily representative of all tissues, whereas arterial blood is essentially uniform and represents the blood drawn from all the tissues indiscriminately. On the other hand, to know exactly what muscle tissue contributes to the chemical changes, the blood supplying and leaving the area in question must be compared.

Practically all experiments show a fall in the amide nitrogen content of arterial blood 20 minutes following the 2 minutes of running. The return to the preliminary resting value is not al-

TABLE III.

Effect of Exercise on Amide Nitrogen of Blood.

Period of exercise, 2 minutes.

Time after running.	Blood amide N.			Time after running.	Blood amide N.		
	Femoral.		Venous – arterial.		Femoral.		Venous – arterial.
	Artery.	Vein.			Artery.	Vein.	
Dog 4, male; age 2 yrs., wt. 16.8 kilos.				Dog 28, female; age 1½ yrs., wt. 13.85 kilos.			
min.	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg.*	*min.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg.*
Preliminary.	162	164	+2	Preliminary.	155	151	−4
3	163	166	+3	3	147	149	+2
10	163	167	+4	10	146	151	+5
20	158	158	0	20	150	150	0
40	156	164	+8	40	155	155	0
60	163	157	−6	60	162	160	−2

ways complete at the end of 60 minutes, but is sometimes accomplished at the end of 40 minutes.

Throughout the experiments recorded in Tables III to VIII, the arterial and venous samples are those from the femoral vessels. By subtracting the arterial value from the venous value one obtains the extra number of mg. of amide nitrogen coming from muscular tissue *via* the vein as compared with the amount entering it in arterial blood.

The experiment with Dog 4, recorded in Table III, shows a result that is typical of a very large proportion of the experiments.

The absolute values for the amide nitrogen in this particular case are near the upper limit of the variations we have found in the normal dog.   After 2 minutes of running there is only a very slight immediate change in the arterial and venous values, indicating, as all the experiments do, a tendency to an increase in amide nitrogen leaving muscle at the time of lactic acid excess.   The characteristic fall in the arterial value occurs here at 40 minutes after exercise, and at this time the venous blood contains an excess of 8 mg. over the arterial value.

The experiment with Dog 28 (see Table III) shows the typical fall in the amide value for arterial blood at 10 minutes after the period of exercise.

The preliminary values here for artery and vein show the vein to contain 4 mg. less of amide nitrogen than are found in the corresponding artery.   To establish equal values for artery and vein would mean, therefore, that the tissues of the leg must give up 4 more mg. of amide nitrogen per 100 cc. of blood.   Not only was this accomplished, but at 10 minutes after exercise the blood of the femoral vein contained 5 more mg. than did the artery at the same instant.   It is important to note that appreciable amounts of amide nitrogen must be involved in such experiments, because the greatly increased rate of blood flow after exercise operates in the direction of obscuring the detection of such outpourings of amide nitrogen.   A given amount of any chemical substance coming from a muscle after exercise is poured into quantities of blood that are larger in amount after exercise than pass through the muscle at rest, and as the amount of blood flowing through the muscle increases, the amide nitrogen leaving the muscle has correspondingly less and less effect upon the composition of the blood taken at any instant.   At the end of 60 minutes the difference between the values for artery and vein has returned to essentially what it was in the preliminary period.

Two control experiments are recorded in Table IV.   We have here duplicated all the features of an exercise experiment except the exercise.   It is seen that when a dog is put through the procedure of such an experiment, with 2 minutes of rest substituted for the period of exercise, there is no noteworthy change in the values obtained by subtracting arterial from venous values for the amide nitrogen of blood.   There is no drop in arterial values as in the

TABLE IV.

Control Experiment; Duplication of All Features of Exercise Experiment except Exercise.

Period of rest, 2 minutes, to correspond to period of exercise.

Time after rest.	Blood amide N.			Time after rest.	Blood amide N		
	Femoral		Venous − arterial.		Femoral.		Venous − arterial.
	Artery.	Vein.			Artery.	Vein.	
Dog 27, male; age 1 yr , wt. 21.5 kilos.				Dog 28, female; age 1½ yrs , wt 13.85 kilos.			
min	*mg per 100 cc*	*mg per 100 cc*	*mg*	*min.*	*mg. per 100 cc.*	*mg per 100 cc*	*mg.*
Preliminary.	150	152	+2	Preliminary.	154	150	−4
3	149	151	+2	3	155	150	−5
10	153	150	−3	10	154	152	−2
20	150	150	0	20	155	153	−2
40	151	150	−1	40	155	155	0
60	152	152	0	60	156	155	−1

TABLE V.

Effect of Exercise on Amide Nitrogen of Blood.

Period of exercise, 2 minutes.

Time after running	Blood amide N.			Time after running.	Blood amide N.		
	Femoral		Venous − arterial		Femoral		Venous − arterial
	Artery	Vein			Artery	Vein.	
Dog 5, male; age 1 yr , wt. 12 7 kilos.				Dog 9,* male; age 1½ yrs., wt. 15.8 kilos.			
min.	*mg per 100 cc*	*mg per 100 cc*	*mg*	*min*	*mg. per 100 cc.*	*mg per 100 cc.*	*mg.*
Preliminary	155	149	−6	Preliminary.	155	151	−4
3	148	153	+5	3	152	152	0
10	153	151	−2	10	148	149	+1
20	144	155	+11	20	144	149	+5
40	152	145	−7	40	145	153	+8
60	149	150	+1	60	147	144	−3

* Urinary NH_3-N for first 20 min. = 7.8 mg. or 23.4 mg. NH_3-N per hr.
last 40   9.4   14.1

exercise experiments, and there is not the slightest tendency toward an outpouring of amide nitrogen from the muscle.

The results in Table V, with Dog 5, show a response that is qualitatively similar to the ones already discussed.   The fall in arterial value occurs here at 20 minutes, as in most of the experiments.   In this experiment the amounts of amide nitrogen leaving the muscles of the leg are considerable.

The experiment on Dog 9 (see Table V) shows the typical response.   The low point in the arterial value is again shown at 20 minutes, with a rather slow return to normal.   The difference between arterial and venous values shows that after muscular exercise there is an outpouring of amide nitrogen from muscle.

Since this dog is one of our best trained normal dogs and is used to lying quietly on the table for the purpose of being catheterized, we obtained all the urine excreted during the 60 minutes after exercise in order to study the ammonia elimination.   The bladder was emptied and washed at the start; two samples were obtained after exercise, one at 20 minutes and the other at the end of 60 minutes.   In both instances the bladder was washed and the washings added to the sample in question.

The findings for urinary ammonia are in accord with the results of Liljestrand and Wilson (2).   During the first 20 minutes the urinary ammonia elimination was at the rate of 23.4 mg. of ammonia nitrogen per hour, and during the following 40 minutes it was at the rate of 14.1 mg. per hour.   Since these results were in agreement with the very complete and convincing work of Liljestrand and Wilson (2), no other urinary analyses were attempted.

To test the effect of acid administration preliminary to the experiment, the following experiment was performed.   Hydrochloric acid was administered for 4 days before a dog was exercised. During the 4 preliminary days, the dog received 350 cc. of $N/7$ acid by stomach tube twice daily (with the exception of the 2nd day, when only half that amount was given).   Therefore, 2500 cc. of $N/7$ acid were given in all.   If this excess of acid appreciably diminished a somewhat fixed supply of ammonia or its precursor in muscle, the lactic acid formed in exercise should, it seems, be neutralized almost wholly by fixed base, and the amount of amide nitrogen given up by muscle should be less.

The results shown in Table VI are entirely different from any-

thing else we have obtained. This is the only exercise experiment in which there is not a single sample of venous blood that has more amide nitrogen in it than there is in the corresponding arterial sample. Both arterial and venous samples show the usual fall in value at 20 minutes after exercise, with a return within an hour.

A further test of the hypothesis that ammonia supplies may be exhausted by unusual demands upon them for acid neutralization, lies in exercise experiments of longer duration in which larger amounts of lactic acid are formed.

TABLE VI.

Effect of Exercise on Amide Nitrogen of Blood, after Acid Administration.
Period of exercise, 2 minutes.

Time after running	Blood amide N.		
	Femoral.		Venous — arterial.
	Artery.	Vein.	
Dog 8, male; age 1½ yrs., wt. 30.3 kilos.			
min.	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg*
Preliminary.	160	160	0
3	159	157	−2
10	157	157	0
20	150	147	−3
40	158	154	−4
60	157	153	−4

Accordingly, Dog 21 was made to run continuously for 5 minutes, at the end of which time it was well fatigued. The results for Dog 21 in Table VII show that after the characteristic drop in arterial amides at 20 minutes after exercise there is a feeble rise, followed by a pronounced fall at 60 minutes. The total drop in the case of the arterial blood is from 154 to 134, and in the case of the venous blood from 151 to 139 mg. This result is in agreement with what might have been predicted from our original hypothesis. We have never had a similar result in any experiment where a 2 minute exercise period was used.

In order to apply a more extreme test, a 10 minute exercise period was tried. The dog was made to run continuously for 10 minutes.

The results with Dog 22 in Table VII are of the same character as with the 5 minute period of running, and the effects are more

TABLE VII.

Effect of Exercise on Amide Nitrogen of Blood.

Time after running.	Blood amide N.			Time after running.	Blood amide N.		
	Femoral.		Venous − arterial.		Femoral.		Venous − arterial.
	Artery.	Vein.			Artery.	Vein.	
Dog 21, male; age 1 yr., wt. 21.9 kilos. *5 min.* exercise.				Dog 22, male; age 1 yr., wt. 20.2 kilos. *10 min.* exercise.			
min.	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg.*	*min.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg.*
Preliminary.	154	151	−3	Preliminary.	141	139	−2
3	155	151	−4	3	146	152	+6
10	155	156	+1	10	142	145	+3
20	149	154	+5	20	137	144	+7
40	Lost.	140		40	126	128	+2
60	134	139	+5	60	125	126	+1
				90	129	127	−2

TABLE VIII.

Effect of Exercise on Amide Nitrogen of Blood in a Dog with Alkali Plethora. Period of exercise, 2 minutes.

Time after running.	Blood amide N.		
	Femoral.		Venous − arterial.
	Artery.	Vein.	
Dog 12, male; age 2 yrs., wt. 18.8 kilos.			
min.	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg.*
Preliminary.	150	156	+6
3	157	158	+1
10	149	158	+9
20	157	152	−5
40	156	155	−1
60	158	151	−7

marked.   This is to be expected from the heavier demands made upon the organism in the production of very excessive amounts of lactic acid.

The results in the experiment with a 5 minute period of running led us to suspect that the fall in blood amides would be greater in amount and the effect more prolonged. Accordingly, samples were taken at 90 minutes after exercise in addition to the regular samples. This is the only experiment in which samples were taken after 60 minutes.

The experiment on Dog 22 fits in with the interpretation which has been advanced for the changes in blood amides that have been observed to occur after periods of muscular exercise. The values for the amide nitrogen of blood not only fell precipitously, but the sequence of the changes is orderly and in the expected direction. Further, amide nitrogen is demonstrated as leaving muscle in quantities sufficient to strengthen substantially the view that ammonia is one of the alkalies that neutralize lactic acid formed in muscular contraction.

Two exercise experiments have been performed on dogs which had received sodium bicarbonate. The urine in these cases was tested and found to be neutral or alkaline during the 60 minutes after exercise.

The experiment on Dog 12, recorded in Table VIII, illustrates the effect of alkali plethora on the results obtained after exercise. Amide nitrogen is increased in the blood, and there is no subsequent fall in the values to levels lower than in the preliminary resting samples.

DISCUSSION.

The foregoing experiments are presented for the interest they may have in connection with what is known of the chemistry of muscular contraction. Since it has been demonstrated that tissues possess the function of converting ammonia nitrogen into amide nitrogen, it follows that the ammonia metabolism may be investigated by studying variations of the amide nitrogen of blood. For the first time, too, it would seem that ammonia may be present in muscle in amounts large enough to play a significant part in acid neutralization.[1]

[1] Parnas, Mozolowski, and Lewinski (12) have studied the ammonia of blood after exercising the arm. In twelve cases they found an average increase of 0 08 mg. of ammonia nitrogen per 100 cc. of blood for the exercised arm. If one calculates the total increase in ammonia nitrogen involved,

The positive demonstration that tissues convert ammonia nitrogen into amide nitrogen indicates that the variations in amounts of ammonia are reflected in the values for the amide nitrogen of blood. Aside from the evidence presented, there is considerable plausibility for such a view, especially when it is remembered that ammonia itself is toxic, and the conversion of metabolic ammonia into amide becomes another example of what has been termed a "protective synthesis."

There is little doubt that, compared with fixed base, ammonia plays a less important rôle in the neutralization of lactic acid formed in muscular exercise. The evidence for the view that ammonia does play a part in the neutralization of lactic acid might be as follows.

In the first place, a comparison of the values for arterial and venous blood from an active tissue area might show that there is more amide nitrogen in venous than in arterial blood. A consideration of the quantities involved makes it seem that such differences might be difficult of detection. The increased rate of blood flow through the muscles after exercise lessens the chance of finding the increases in the amount of amide nitrogen that may leave a tissue when lactic acid is being neutralized. On the other hand, it is well known that in resting tissue a very considerable area of the vascular bed is virtually closed to the circulating blood, and opens up when the tissue is stimulated to increased activity. The extent to which this happens in an active muscle with an increased blood supply after exercise increases the amount of reserve material that can be called upon for such an emergency and might materially affect the chemical composition of blood from the femoral vein. A large series of experiments has been carried out to see if such differences exist.

We know that when ammonia is injected into a muscle (*via* the femoral artery) there is an increased output of amide nitrogen

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a 60 kilo person having 3 kilos of blood would have an increased amount of ammonia nitrogen of 2.4 mg. Obviously less than one-half of the person's blood is passing through the exercised arm, and so the increase in question must involve an amount of ammonia nitrogen of the magnitude of 1 mg. Such an amount of ammonia cannot have any significance in the neutralization of lactic acid, even if one supposes, as now seems unwarranted, that these low values really represent circulating ammonia.

from the muscle, indicating amide synthesis from ammonia. There is evidence that tissues contain ammonia, and when normal dogs are exercised, and lactic acid is poured into the blood stream, there appear, simultaneously, increased amounts of amide nitrogen in the blood. Since the introduction of ammonia into muscle is followed by increased amide output from the muscle, it seems justifiable to assume that a similar outpouring of amide nitrogen from muscle after exercise is to be attributed to ammonia that has come from muscle with the outpouring of lactic acid.

The unknown material from which ammonia is formed in muscle may be termed an ammonia precursor. When ammonia is used for acid neutralization in muscle, ammonia or this unknown precursor must diminish in amount corresponding to the quantity of ammonia so used. During the following period the organism is faced with the situation of readjusting itself with regard to the redistribution of fixed base and the reestablishment of the quantities of ammonia, or its precursor, that represent resting values.

A dog on the acid régime (either the normal dog or one that has had HCl administered by mouth) shows a reduced value for the amide nitrogen of arterial blood at 20 minutes after exercise. Two explanations of this suggest themselves. In the first place, since this is the time of surplus ammonia excretion the fall in the amide nitrogen of blood may be due, in part, to the excretion of ammonia. From an analysis of the data it seems probable that the amount of ammonia excreted can explain hardly more than a part of the fall in the amide nitrogen content of arterial blood at 20 minutes after exercise. Further, it is known that at this time the lactic acid is well disposed of, leaving an excess of base available for the ordinary acid neutralization requirements, and the lower value for blood amide nitrogen may indicate a restocking of the tissues with ammonia (or its precursor) which was "washed out" by the initial outpouring of lactic acid. As the muscles approach recovery (40 to 60 minute period) they are becoming more completely stocked with ammonia precursor so that less and less of it is therefore withdrawn by the tissues for the reestablishment of their normal resting amounts, and ammonia again takes its usual share of the acid neutralization burden, and is again reflected in blood in the normal concentration for blood amides. This reciprocal arrangement between fixed base and ammonia, whereby the temporarily ex-

cessive excretion of one of them is compensated for latterly by correspondingly larger amounts of the other, is too well known to require further comment.

Further study of changes in the amide nitrogen coming to and leaving other areas of the body are necessary before one can confidently interpret the fall in arterial amides observed in such exercise experiments.

At present there seem to be no facts which stand in the way of the accompanying scheme, which is offered to represent tentatively a possible way of considering the mode of transport of ammonia through the blood from the tissues, where it is formed, to the kidneys, where it is excreted.

Reaction No   Site.

1. Tissue. Ammonia + lactic acid → ammonium lactate.

2. Tissue. $\frac{Blood}{protein}$|—COOH + NH$_4$$^+$· ›$\Big|\frac{Blood}{protein}$| —COONH$_4$ — H$_2$O ·

$\frac{Blood}{protein}$ ·CONH$_2$ (amide)

3. Blood. Lactate ion + NaHCO$_3$ → CH$_3$CHOHCOONa + H$_2$CO$_3$

4. Kidney. $\frac{Blood}{protein}$|—CONH$_2$ + HOH—deamidase · ›$\Big|\frac{Blood}{protein}$|—COOH + NH$_3$

CH$_3$CHOHCOONa + H$_2$CO$_3$ → CH$_3$CHOHCOOH + NaHCO$_3$

5. Urine.   · Ammonium lactate

SUMMARY.

1. Tissues possess the function of detoxifying ammonia by the synthesis of amides of the blood proteins. This is shown by the fact that after a dog has recovered from the poisoning caused by the intravenous injection of ammonium carbonate solutions, it

can receive a similar amount of the solution *via* the femoral artery without any toxic symptoms, and in both instances there is a rise in the amide nitrogen content of the blood. The order of injection is immaterial.

2. Dogs with normal kidneys quickly reduce the higher level of blood amide nitrogen that is caused by the injection of ammonium carbonate solutions.

3. It has been demonstrated that the tissues of the leg of the dog possess the power of rapidly synthesizing amides from injected ammonia.

4. Short periods of muscular exercise are followed at 20 minutes by a moderate lowering of the amide nitrogen content of arterial blood, with a return to normal within an hour. This lowering is considered to be caused in part by the excretion of extra ammonia at this time and also by the relative alkali plethora which follows the resynthesis of the lactic acid precursor in muscle.

5. Comparison of simultaneous samples of blood taken from the femoral artery and the femoral vein before and after exercise show that after exercise the venous blood contains more amide nitrogen than the arterial blood. The increases in amide nitrogen content are as high as 7.6 per cent with a method that has been shown to be accurate to within less than 1 per cent.

6. The results are in harmony with the view that ammonia plays a part in the neutralization of acids formed in muscular exercise, the ammonia, so used, appearing in the blood of the femoral vein not as an ammonium salt, but as an amide of blood protein.

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THE INFLUENCE OF CHOLINE UPON BLOOD SUGAR CONTENT.

By FRANK P. UNDERHILL AND JOSEPH PETRELLI.

(From the Department of Pharmacology and Toxicology, Yale University, New Haven.)

The wide-spread distribution of choline in the cells of plants and animals either as choline itself or in the form of its precursor, lecithin, carries with it the possibility of a specific function in metabolic processes. Its toxicity is relatively low (1). On the other hand its introduction into the blood causes a fall of blood pressure (2). It possesses a stimulant effect on isolated muscle of the intestine, uterus, and iris and causes an increased secretion of the lacrimal, salivary, and sweat glands Saliva is the secretion most easily induced.

Dresel and Zemmin (3) made the interesting observation that choline administered in 0.5 gm. doses to normal man either *per os* or subcutaneously causes a marked decrease in blood sugar content. Moreover, in diabetic patients choline in the same dosage induces a condition of hypoglycemia. Employing rabbits Madinaveitia and Hernandez (4) assert that small doses of choline induce hypoglycemia whereas larger doses call forth hyperglycemia.

These statements in the literature relative to the influence of choline upon blood sugar content justify an extended investigation into the mechanism involved in the reaction. We have tested the influence of varying doses of choline upon the blood sugar content of the dog and rabbit. Our results are not in harmony with those of previous investigators since with doses of choline sufficient to produce an effect upon blood sugar content hyperglycemia was always in evidence. Hypoglycemia was never observed.

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EXPERIMENTAL.

Methods.

The experimental animals (rabbit and dog) received no food 24
hours previous to and during the period of experimentation
although they had free access to water. Blood was drawn from a
marginal ear vein and blood sugar was estimated by the method of
Folin and Wu. Choline as the hydrochloride was always adminis-
tered subcutaneously. In Table II figures for blood sugar content
are not given beyond the 24 hour limit. Data are available,
however, up to a period of 72 hours but inasmuch as they are
uniformly negative they have been omitted from Table II.

TABLE I.

Influence of Fasting on Blood Sugar Content in Rabbit.

Date.	Rabbit No.	Weight.	Hrs of fasting.					
			24	25	26	27	28	29
			Sugar per 100 cc. blood.					
1924		*gm.*	*mg*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Dec. 5	1	2080	130	141	135	131	140	
" 5	2	2460	123	127	133	134	130	
" 12	3	2520	116	116	121	125	115	121

Hemoglobin estimations, according to the Newcomer method
were made at periods corresponding to the blood sugar determina-
tions. Inasmuch as these figures did not deviate from the normal
range they are omitted from the tables.

Experiments with Rabbits.

Influence of Fasting on Blood Sugar Content of Rabbit.—Control
experiments were made on rabbits that had fasted for 24 hours.
It is apparent from Table I that the hourly changes in blood sugar
content under these experimental conditions are too small to be of
significance.

*Influence of Varying Doses of Choline upon Blood Sugar Content
in Rabbit.*—In the experiments with rabbits doses of choline
varying from 0.025 to 0.4 gm. per kilo of body weight were em-

ployed. It is evident from Table II that the blood sugar content was not significantly affected by a dose of 0.025 gm. per kilo of choline.

The same statement is pertinent for a dose of 0.1 gm. per kilo of choline. When, however, a dose of 0.2 gm. per kilo of choline was given a transitory but marked hyperglycemia was in evidence. 15 minutes after choline injection salivation occurred which was soon followed by lacrimation and diarrhea. These symptoms were

TABLE II.

Influence of Choline Hydrochloride upon Blood Sugar Content.

Body weight.	Choline HCl injected.	Blood sugar before injection.	Blood sugar content after choline HCl injection.								
			½ hr.	1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	6 hrs.	7 hrs.	24 hrs.
						Rabbit.					
kg.	*gm. per kg.*	*mg. per kg.*	*mg. per kg.*	*mg. per kg.*	*mg. per kg.*	*mg. per kg.*	*mg. per kg.*	*mg. per kg.*	*mg. per kg.*	*mg. per kg.*	*mg. per kg.*
2.4	0.025	146	131	132	149	141		141			130
2.2	0.100	125	142	140	140	134	133	134		127	126
2.1	0.200	139	187	205	221		149		139		128
2.2	0.300	114		381	444	270	176	131			125
2.5	0.400	113		179	206*						
2.4	0.400	116		185	247*						
3.2	0.100†	133	182	286	308			148			129
						Dog.					
10.6	0.025	110	114	113	110	101	104		111	92	105
6.5	0.100	114	130	114	118	118		115		112	117
6.5	0.200	111	141	154	143	118	122	118	112		118
5.1	0.300	93	119	131	123	109	116	111	114	114	100

* Died in convulsions.

† Two injections of 0.100 gm. per kilo each at intervals of ½ hour.

not severe but persisted for 1½ hours; then began to subside and had disappeared by the 4th hour after the injection.

After a dose of 0.3 gm. per kilo of choline the blood sugar content of the rabbit showed a marked increase which, however, rapidly regained a normal level. In spite of the marked hyperglycemia glycosuria was absent.

Notable signs of choline poisoning, salivation, lacrimation, and diarrhea, were in evidence soon after the injection of choline.

These symptoms gradually became less noticeable until at the end of the 5th hour the animal appeared normal.

A dose of 0.4 gm. per kilo of choline proved fatal to the two animals tested. Both were dead in $2\frac{1}{4}$ hours after the injection, death resulting from convulsions and respiratory failure. In both instances a marked hyperglycemia was observed.

Influence of Repetition of a Small Dose of Choline upon Blood Sugar Content in Rabbit.—In this experiment the rabbit was given two doses of 0.1 gm. of choline per kilo at an interval of $\frac{1}{2}$ hour (see Table II). From the table it is evident that hyperglycemia is augmented by the second injection and that the period of high blood sugar is prolonged. Symptoms characteristic of choline poisoning were present but were not marked.

Experiments with Dogs.

The experiments with dogs were carried on in the same manner as those with rabbits. Doses of choline hydrochloride varying from 0.025 to 0.3 gm. per kilo of body weight were employed.

From Table II it may be observed that doses of 0.025 to 0.3 gm. per kilo of choline were without significant influence upon the blood sugar content of the dog. The values obtained were all within the normal range for this animal. With the larger doses symptoms of choline poisoning were in evidence.

It is quite apparent that the dog is much more resistant than the rabbit to the action of choline in altering blood sugar content.

CONCLUSIONS.

Choline in suitable doses subcutaneously administered to rabbits causes a significant hyperglycemia without evidence of glycosuria. Contrary to previous investigators a hypoglycemia was never encountered.

Comparable doses of choline similarly injected into dogs fails to alter appreciably the blood sugar content.

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IS THERE A RELATIONSHIP BETWEEN THE SPLEEN AND CALCIUM METABOLISM?

By FRANK P. UNDERHILL AND ERWIN G. GROSS.

(From the Department of Pharmacology and·Toxicology, Yale University, New Haven.)

In 1925 Hall and Ablahadian (1) stated that after the removal of the spleen the blood calcium of rabbits decreased almost 50 per cent but returned to normal after intravenous injection of spleen extract (nucleoprotein).   Moreover, the administration of splenic extract to normal rabbits caused a greater increase of blood calcium than did an extract of parathyroid.   Krumbhaar (2) in reference to the work of Hall and Ablahadian remarks that "Unfinished work of our own tends to confirm them to a certain extent."

These statements are so suggestive of a possible rôle of the spleen in calcium metabolism as to warrant further investigation. Accordingly an attempt has been made to repeat the experiments of Hall and Ablahadian.   Since the details of their work are given only incompletely it is uncertain whether our experiments can be regarded as duplicates of theirs.   It is possible that this fact may account for our failure to confirm their results.   Under our experimental conditions removal of the spleen does not influence the level of serum calcium in rabbits nor is the serum calcium notably affected by intravenous injections of spleen nucleoprotein.

Influence of Splenectomy upon Serum Calcium.

Full grown male rabbits maintained upon a diet of carrots and oats were splenectomized under urethane-ether anesthesia and serum calcium (3) content was determined for several days after the operation.   An illustrative protocol of these experiments is given in Table I.

Influence of Intravenous Injection of Spleen Nucleoprotein upon Serum Calcium of Normal Rabbits.

Full grown male rabbits were maintained upon a diet of carrots and oats and a 5 per cent solution of spleen nucleoprotein was

TABLE I.

Experiment 1.

Rabbit, 2.1 kilos.

Date.	Serum Ca.	Remarks.
1927	*mg. per 100 cc.*	
Oct. 14	11.7	Normal.
" 17		Spleen removed at 2.30 p.m.
" 19	12.1	
" 21	12.2	
" 24	12.0	
Nov. 2	12.1	
" 8	11.9	

TABLE II.

Experiment 2.

Rabbit, 2.2 kilos.

Date.	Serum Ca.	Remarks.
1927	*mg. per 100 cc.*	
Nov. 7, 10.30 a.m.	12.1	Normal.
" 7, 10.30 "		Injected 1 cc. 5 per cent spleen nucleo-protein.
" 8, 11 "	12.0	
" 8, 11 "		Injection of nucleoprotein as above.
" 9, 11 "	11.9	
" 9, 11 "		Injection of nucleoprotein as above.
" 10, 2 p.m.	12.0	
" 10, 2 "		Injection of nucleoprotein as above.
" 11, 2 "	11.7	
" 11, 2 "		Injection of nucleoprotein as above.
" 12, 2 "	12.2	

injected once daily into an ear vein. The nucleoprotein was pre-pared from pig spleen, obtained from the abattoir, by extraction with sodium chloride solution and precipitation with acetic acid. The precipitate was washed with alcohol and ether and dried.

Solution was effected with 0.9 per cent sodium chloride solution. An illustrative protocol of these experiments is given in Table II.

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DENATURATION OF INSULIN PROTEIN BY CONCENTRATED SULFURIC ACID.

By FRITZ BISCHOFF and MELVILLE SAHYUN.[*]

(*From the Chemical Laboratory of the Potter Metabolic Clinic, Santa Barbara Cottage Hospital, Santa Barbara.*)

In a former paper from this laboratory (1), it was shown that insulin could be treated with very dilute nitrous acid in aqueous solution without destroying its potency. In such preparations the NaOH labile sulfur of Abel (2) was considerably reduced and under certain conditions the product was insoluble at an acid pH. These preparations gave a strong biuret test. Scott (3) tried the action of strong nitrous acid in acetic acid upon insulin. All the potency was destroyed. The biuret test was positive. Since the biuret color reaction requires one free acid amide group and a neighboring substituted or unsubstituted acid amide group or free α-amino group capable of functioning as a substituted acid amide group through lactam formation, it should be theoretically possible to render a protein substance, like insulin, biuret-free by destroying the amino and amide groups with nitrous acid. It is common practice in working with aromatic compounds which are difficult to diazotize, to resort to solution in concentrated H_2SO_4 and to diazotize in this medium. It appeared highly improbable to us that insulin would be able to withstand such a treatment with-

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[*] The insulins used in these studies and their denatured products were evaluated for potency by Mr. Sahyun according to the method developed in this laboratory (Sahyun, M., and Blatherwick, N. R., *Am. J. Physiol.*, **76**, 677 (1926)). In a former communication from this laboratory, Lilly's iletin U-80, 80390-758690 was used as the standard for the assay. Insulin, purified by Abel's crystallization method, assayed 55 to 60 units per mg. on the basis of this standard. More recent batches of iletin are 25 per cent stronger by our method of assay than batch U-80, 80390, *etc.* In this paper we have adopted a standard 25 per cent more powerful than the old standard. This values the potency of the crystalline insulin as 35 to 40 units per mg., the strength now assigned to it by Abel.

TABLE I.

Treatment of Insulin with Concentrated Sulfuric Acid and with Various Reagents in Concentrated Acid.

Starting material.	Treatment.	Product.		
		Potency.	Biuret.	Millon.
		units		
Crystalline insulin 900 units, 40 per mg.	Solution in 3 cc. H_2SO_4 at $-10°$. Washed twice.	450	++	
" 1100 " 37 " "	Solution in 0.5 cc. acetic acid. Add 1.25 cc. H_2SO_4 at 5°. Washed five times.	600 (25 per mg.)	++	
3000 units, 28 per mg.	"	1300	++	
Crystalline insulin 3500 units, 35 per mg.	Solution in 0.5 cc. acetic acid. Add 2.0 cc. H_2SO_4, 10 mg. nitrite. 1 hr. at 5°. Washed four times.	1600	–	
" 2800 " 28 " "	Solution in 0.4 cc. acetic acid. Add 1.5 cc. H_2SO_4, 25 mg. nitrite. Crystal $CuSO_4$. 16 hrs.	None.	–	
2000 units, 20 per mg.	Solution in 0.5 cc. acetic acid. 2.5 cc. H_2SO_4, 10 mg. nitrite. 5 min. at 5°.*	800	+	
700 " 7 " "	Solution in 0.5 cc. acetic acid. 2.0 cc. H_2SO_4, 20 mg. nitrite. 5 min. at 5°.*	500	+	
1900 " 18 " "	Solution in 3 cc. H_2SO_4. 0.1 gm. nitrite, 0.2 gm. $(NH_4)_2SO_4$. 30 min. at $-10°$.*	650	–	
1900 " 18 " "	Solution in 3 cc. H_2SO_4. 10 mg. nitrite. 30 min. at 5°.*	200	–	++
1900 " 18 " "	Solution in 5 cc. acetic acid. 20 mg. nitrite. " 0.5 " " 2 cc. H_2SO_4, 25 mg. nitrite. 3 hrs. at 5°.	600	++	++
Crystalline insulin 2000 units, 28 per mg.		800	–	+

Crystalline insulin 800 units, 40 per mg.	3 cc. H_2SO_4 at $-10°$. 2 drops dimethyl sulfate.	300	+	
1800 units, 18 per mg.	2 cc. H_2SO_4 at 5°. 50 mg. NaCN.	1100	++	
1600 " 18 " "	2 " " Add insulin at 5°. 0.1 cc. 40 per cent HCHO.	None.	+	−
1000 " 30 " "	4 cc. H_2O. Add insulin at 5°. 0.1 cc. 40 per cent HCHO.	500	+	+

* The reaction mixture was poured upon ice mixed with bicarbonate.

out total loss of potency.   A trial showed, however, that over half
the potency was recovered.   In this paper are recorded the results
of a series of experiments in which insulin was treated with various
reagents in concentrated H_2SO_4 solution.

Concentrated Sulfuric Acid.

The action of concentrated H_2SO_4 alone is of interest.
Powdered insulin dissolves in concentrated H_2SO_4 slowly at $-5°$
to $+5°$.   When the solution is poured upon chipped ice, an
insoluble precipitate results.   This product is insoluble at a pH
more acid than 4.8.   It possesses no isoelectric point and retains
half the original potency.   The benzoylated insulins of Scott and
of this laboratory and the acetyl insulin of Jensen and Geiling (4)
are also acid-insoluble, but the potency of these preparations is
less than a fifth of the original.   The rate of solution of insulin,
as for most organic compounds, is very slow in concentrated H_2SO_4.
In dissolving vat dyes in H_2SO_4, it has been found possible to
accelerate the rate of solution by first powdering the dye with
anhydrous sodium sulfate.   This procedure was attempted for
insulin without success.   Since insulin dissolves rapidly in warm
acetic acid, solution in this solvent with subsequent addition of
the H_2SO_4 was tried.   The acetic acid solution of the insulin was
frozen and cold sulfuric acid then added.   In this manner com-
plete solution was effected in the H_2SO_4 in a few minutes.   The
resulting product has the same properties as the one obtained by
using H_2SO_4 alone.   See Table I.

Nitrite in Concentrated Sulfuric Acid.

When nitrite is added to the H_2SO_4 solution of insulin, a
denatured product with about one-third the original potency
results.   There is no isoelectric point.   It is insoluble below pH
4.8.   Above this pH to neutrality, a nearly colorless aqueous
solution results.   Above pH 8.0 the solution is yellow, indicative
of some nitrosophenolic and possibly other chromophoric forma-
tions.   In four instances the acid-insoluble insulin gave a negative
biuret ring test.   The yellow-colored solution at the alkaline
pH tends to mask the biuret color, when the test is made in the
conventional way by adding 1 drop of $1:1000$ $CuSO_4$ to 50 units
of insulin in strongly alkaline solution in a volume of 1 cc.   By

adding an excess of $CuSO_4$ and centrifuging down the blue precipitate, a faint blue coloration was obtained for the insulins which gave a negative ring test and an intense purple color for those which gave positive ring tests. Biuret-positive insulin of equal potency and nitrogen content added to the biuret-free insulins in either the ring or solution tests gave a positive reaction. The alkaline solutions which gave negative biuret tests become positive upon standing several hours. An aqueous solution of one of the biuret-free insulins, which had stood at the neutral pH for a week, also became positive, indicative of hydrolysis or rearrangement to give the groups essential for the biuret test. The results are summarized in Table I. We are unable to give directions which will produce a biuret-negative insulin every time. Our experience was similar in following Piper, Allen, and Murlin's directions (5) for the preparation of biuret-free insulin by perfusion. We now regard Murlin's biuret-free insulin as a denatured protein. His process of purification, continued extraction at a more or less elevated temperature with acid alcohol, would give rise to conditions suitable to hydrolysis and alkylation, both reactions capable of destroying the linkages essential to the test. In the diazotization in H_2SO_4 with subsequent dilution in water, hydrolysis with the liberation of acid amide groups is possible. The hydrolysis might take place when the concentrated acid is diluted and so negative the work of the nitrous acid.

Nitrogen determinations by combustion were made for a crystalline insulin, a crystalline insulin which had passed through the concentrated H_2SO_4 treatment and a crystalline insulin which had been diazotized in concentrated H_2SO_4. These products were powdered and dried in a vacuum desiccator over $CaCl_2$ before analysis. The value of 15.6 per cent for the crystalline insulin is in good agreement with the values of 15.72 and 15.68 obtained by Wintersteiner, du Vigneaud, and Jensen (6) for their crystalline insulin. The lower value (13.7 per cent) for the nitrite-treated insulin as compared with 14.5 per cent for the product denatured by H_2SO_4 was rather to be expected, even in the presence of some nitrosylation. Since no sulfur determinations were made, it is not possible to speculate as to whether the action of the H_2SO_4 is one of sulfonation, dehydration, or rearrangement.

Analyses for Dumas Nitrogen.

I. Crystalline insulin. 73.8 mg. gave 10.4 cc. N_2 at 26° and 759 mm. 15.6 per cent N.

II. Sulfuric acid-treated insulin. 49.2 mg. gave 6.4 cc. N_2 at 25° and 756 mm. 14.5 per cent N.

III. Nitrite-sulfuric acid-treated insulin. 70.1 mg. gave 8.75 cc. N_2 at 29° and 755 mm. 13.7 per cent N.

Insulin, which is denatured by concentrated H_2SO_4, is not reactivated by standing in dilute alkaline solution. Two preparations were permitted to stand at pH 8.5 for 36 hours, without any change of potency.

Cyanide and Dimethyl Sulfate in Sulfuric Acid.

The addition of small quantities of NaCN or dimethyl sulfate to the H_2SO_4 was found to have no effect upon the inactivation of the dissolved insulin. Formalin completely inactivates the insulin, yielding a biuret-positive, Millon-negative product. This is interesting because the same concentration of aqueous formalin only partially destroys the potency. The results are given in Table I.

In our experiments insulins of varying degrees of purity, including the crystalline insulin of Abel, were used. The results are essentially the same. It will be noted, however, that the least pure insulin was the most resistant to treatment, over 70 per cent of the potency being recovered. A similar observation was made in our study of the inactivation of insulin by H_2S (1).

We are indebted to Dr. N. R. Blatherwick for some of the crystalline insulins used in our experiments.

SUMMARY.

Concentrated H_2SO_4 at a low temperature dissolves insulin and renders it water-insoluble at a pH more acid than 4.8. Half the potency is retained. Cyanide and dimethyl sulfate in concentrated H_2SO_4 do not augment inactivation, while formalin completely destroys the potency. Nitrite in several instances produced a biuret-free product, retaining half the original potency.

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STUDIES IN THE CHEMISTRY OF HEMOGLOBIN.

III. THE EQUILIBRIUM BETWEEN OXYGEN AND HEMOGLOBIN AND ITS RELATION TO CHANGING HYDROGEN ION ACTIVITY.

BY RONALD M. FERRY AND ARDA ALDEN GREEN.*

(*From the Department of Physical Chemistry in the Laboratories of Physiology, Harvard Medical School, Boston.*)

INTRODUCTION.

Knowledge concerning the combination between oxygen and hemoglobin, which had been obtained prior to 1912, was summarized by Barcroft in his book, "The Respiratory Function of the Blood" (1). To this understanding Hoppe-Seyler and his contemporaries, and later, Barcroft, Haldane, and Hill, contributed notably. Since this book appeared it has been found that the picture presented was too simple and neglected certain other interdependent variables such as hydrogen ion activity, salt concentration, and distribution of water and electrolytes between plasma and cells.

Beginning with the appearance of the paper by Christiansen, Douglas, and Haldane (2), and followed by the papers of Henderson, of Parsons, of Van Slyke and his collaborators—to mention only a few of the investigators involved—data have accumulated rapidly. As a result, we now have in all cases at least a qualitative picture of the equilibria involved. In certain cases, notably that of the acid-base equilibrium, our knowledge is relatively satisfactory, both theoretically and quantitatively; in others, either theory has outstripped precise data, or quantitative data lack a theoretical explanation. None the less empirical relationships

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* Le Conte Memorial Fellow, University of California, 1924–1925, the period in which the experiments herein described were performed. Research Fellow in Medicine, National Research Council, 1927.

have been demonstrated in many instances.   But despite the fact that the equilibria involving hemoglobin, hydrogen ion activity, and oxygen tension have received particular attention, there is as yet no adequate theoretical explanation, nor do satisfactory data correlating these equilibria over a wide range of hydrogen ion activity and percentage saturation of hemoglobin with oxygen exist.

The experiments described in this paper were, therefore, undertaken in order to make such data available, and, if possible, to provide a theoretical interpretation for them.   To this end we have constructed isohydrionic oxygen dissociation curves for the pH range 4.91 to 8.88, derived from experimental values for percentage saturation of hemoglobin at varying oxygen tensions, obtained in heavily buffered solutions of crystallized horse hemoglobin.

Methods.

A description of the methods employed may conveniently be divided into four portions: the preparation of pure hemoglobin; the elaboration of solutions of known hydrogen ion activity from this material; equilibration of the solutions with suitable gas mixtures; and, finally, the analysis of the equilibrated solutions for their oxygen content.

Preparation of Hemoglobin.

Hemoglobin was prepared by a modification of a method previously described by one of us (3).   This method depends on the separation of red blood cells from diluted plasma by means of the Sharples centrifuge, and repeated crystallization of oxyhemoglobin from a cold, concentrated solution, after complete oxygenation and adjustment of the pH to a point slightly more acid than 6.8.

3 liters of horse red blood cells[1] were diluted with 12 liters of 1.5 per cent sodium chloride solution which had previously been cooled to 2°.   The resulting volume of 15 liters was then run

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[1] Obtained from the Massachusetts Antitoxin Laboratory through the courtesy of its director, Dr. Benjamin White.

through a modified bowl[2] of the Sharples laboratory centrifuge rotating at a speed of about 30,000 R.P.M. The bowl, from which the vanes at the lower end had been removed, nearly eliminated hemolysis during centrifugation. Consequently, we could wash the corpuscles once by this procedure. Inasmuch as we used large volumes of salt solution, most of the plasma proteins and salts were separated from the cellular suspension at this stage of the preparation.

In order to prevent possible immediate crystallization the concentrated cells were allowed to run into 100 cc. of distilled water for each liter of cells originally present. Occasionally crystallization did occur even after the addition of distilled water. In this event the suspension was vigorously stirred and 1 N KOH added slowly until the crystals dissolved. Concentrated alkali had to be added with extreme care in order to avoid denaturation and the formation of methemoglobin.

In order to separate hemoglobin from cellular débris the partially laked suspension of erythrocytes was again passed through the Sharples centrifuge. This time the ordinary bowl was used, rotating at 35,000 to 40,000 R.P.M. in order to lake the corpuscles mechanically. Very few cell bodies were then observed microscopically.

By this procedure an initial volume of 3 liters of erythrocyte suspension had been concentrated to about 2 liters of 25 to 28 per cent hemoglobin solution. From this solution oxyhemoglobin could readily be crystallized as rhomboid plates. The factors favoring crystallization are concentration of solution, hydrogen ion activity in the neighborhood of the isoelectric point, low temperature, and complete oxygenation. In order to attain the latter end, the alkaline solutions were stirred by a motor-driven Luther

[2] The bowl, at first used, was devised by W. B. Wescott and patented by the American Protein Products Company, Boston, to whom we are indebted for its use. The essential features are, (1) the elimination of vanes in the bottom of the usual bowl, and (2) the insertion of four vanes half way up the bowl. By means of its device great peripheral velocity is imparted rather gradually to the cell suspension, and the impact of rapidly moving vanes on red cells, which have no peripheral velocity on entering the bowl, is avoided. Hemolysis is largely avoided by this device. We have lately found that merely removing the vanes gives satisfactory results and is much simpler as the bowl does not have to be balanced.

stirrer in the cold room at 2°; the speed of stirring was regulated by a rheostat, so that changes in the viscosity of the liquid were counteracted. The Luther stirrer consists of an open ended T-tube fused to a solid glass rod. About 2 inches above the cross-piece there is another opening into the tube. This device provides effective agitation and complete oxygenation. During stirring the reaction was adjusted to about pH 6.6, either by the gradual addition of $\frac{2}{3}$ M phosphate buffer in an amount sufficient to give final concentration of 0.1 M phosphate, or by the addition, drop by drop, of 0.1 M HCl.[3] Because of irreversible changes inactivating the hemoglobin, this must be done with extreme care. At intervals during the titration, drop samples were placed on microscope slides. A few seconds' exposure to air sufficed to evaporate and concentrate the solution, inducing crystal formation. If rods appeared, the solution was still too alkaline and the titration was continued until a sample revealed rhomboid plates microscopically. After the first appearance of crystals another 5 to 10 cc. of 0.1 N HCl per 500 cc. of solution were added, inducing the rapid formation of crystals readily distinguishable macroscopically by the sheen of the suspension. Too much acid must be avoided since denaturation of hemoglobin occurs very rapidly. This is shown by a color change from red to dark brown. From 160 to 320 cc. of 0.1 N HCl generally sufficed to crystallize oxyhemoglobin in 1 liter of concentrated solution. The rather wide limits are presumably due to differences in initial hydrogen ion and hemoglobin concentrations. The steps of washing the erythrocytes, separation of hemoglobin from the cells, and crystallization could easily be completed in 2 hours.

After crystallization had begun the solution was stirred about 1 hour longer and then allowed to stand in the cold room at least another hour. Centrifugation for 15 minutes in the usual laboratory centrifuge yielded a closely packed crystalline sediment occupying a volume equal to that of the supernatant liquid. Centrifugation was performed either in chilled cups or, preferably, in a centrifuge mounted in the cold room.

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[3] Crystallization can also be induced by the addition of sulfuric, acetic, and phosphoric acids, and presumably by the addition of any acid strong enough to change the pH to 6.6. The shape of the crystals obtained depends on the acid used.

The crystalline hemoglobin was then purified by repeated washing and by recrystallization. Washing was done by mixing the crystals with 0.5 to an equal volume of cold distilled water. The suspension was again centrifuged for 15 minutes. This process could be repeated several times without an undue loss of material. After two washings the salt concentration was reduced to a point negligible in comparison with that of the buffer used in our experiments.

Recrystallization was invariably used when the first crop of crystals was formed in a phosphate buffer, and usually when the crystals were formed by acid titration. To the centrifuged crystals was added about half their volume of water in order to facilitate stirring. The solution was then vigorously stirred and 1 N KOH added drop by drop until solution was complete. Usually 5 cc. of alkali per 100 cc. of crystals sufficed.

Centrifugation of the solution removed any insoluble impurity. 0.1 N HCl equivalent to the KOH used in dissolving the crystals was now added, the same precautions being observed as during the first crystallization. Repeated recrystallization could be performed without undue loss of material.

In order to free preparations from electrolytes and secure crystals of a high degree of purity, a dilute isoelectric solution of hemoglobin was dialyzed under reduced pressure. We used this procedure infrequently since the high concentration of salts used in our experiments made it unnecessary.

The successful preparation of hemoglobin by the method described depends largely on bringing a concentrated solution to a proper pH and the observance of precautions already mentioned, but which, because of their importance, we think it worth while to state again: (1) The avoidance of local or general excess of acid, which leads to cleavage and possibly denaturation. (2) Rapidity of the process. Hemoglobin solutions change to methemoglobin spontaneously on standing. (3) Low temperature maintained at 2° as nearly as possible. Increasing the temperature increases the rate of transformation to methemoglobin. (4) Complete oxygenation.

If, however, these precautions are observed, one can obtain a very pure product as is suggested by ratio of protein determined

by the Kjeldahl method to that determined by oxygen capacity and the iron ratio.[4]

The preparations used in our experiments were made by the hydrochloric acid titration method with the exception of Preparations 21 and 22, which were made by adding phosphate buffer.

Preparation of Buffered Hemoglobin Solutions for Equilibration.

Buffered hemoglobin solutions of desired pH were made by adding to a suspension of oxyhemoglobin crystals phosphate or borate solutions, together with enough alkali to change the pH of hemoglobin to a given value.   The quantities were so chosen that final concentrations of phosphate were 0.167 M, those of borate M/9, and that of hemoglobin 0.001 M.[5] Electrometric measurement indicated that pH of fully reduced solutions deviated from the intended values by less than 0.01 in pH.

The phosphate hemoglobin solutions were prepared by addition to a suspension of oxyhemoglobin crystals of sufficient M KH_2PO_4 and 0.884 M KOH to bring the hemoglobin to the desired pH, and to make the final concentration of phosphate 0.167 M. The amount of KOH necessary to change the hemoglobin and phosphate to the desired pH was determined by reference to titration curves for reduced hemoglobin[6] and buffer.   The final concentration of the hemoglobin was approximately 6.4 gm. per 100 cc. or approximately 0.001 M, on the basis of a molecular weight of 66,800, consistently assumed in this paper.

The pH finally attained depended chiefly on the buffering value of the phosphate and to a slight extent on that of hemoglobin. Whereas the concentration of the former (0.167 M) is relatively large compared with that of the latter (0.001 M, or in terms of the probable number of acid groups affected by oxygenation, 0.004 N), it is evident that the phosphate buffering effect must be correspondingly great.   Moreover, slight errors in the amount of

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[4] Unpublished experiments of one of us.   This subject requires further investigation since the nitrogen factor of Abderhalden on which our observations were based was doubtless made on the basis of dry weights of material prepared by the alcohol-ether method.

[5] Assuming a molecular weight of 66,800.   This value accords closely with the values obtained by Svedberg (4) and Adair (5).

[6] Unpublished data by Cohn and Ferry.

alkali or acid added to reduced hemoglobin produce errors in the pH for the reduced solution of less than 0.01 in pH.   The change in pH attendant upon completely oxygenating the reduced solutions is more serious in its magnitude even in the case of phosphate-buffered solutions and increases with increasing pH.   It can be shown that this shift varies from approximately 0.02 in pH at pH 6.55 to about 0.06 in pH at pH 7.95.   At pH 7.38 the shift is equal to about 0.04 in pH.   It is to be noted that the greater changes in pH occur just where the phosphate buffer system becomes inefficient.   The numerical values given for the change in pH are, of course, maximal, inasmuch as the change is proportionately less with incomplete oxygenation.

At points more alkaline than pH 7.95 we made use of boric acid-borate buffers.   Such buffers are somewhat less satisfactory, largely because considerable uncertainty exists concerning the precise mechanism of their action.   Because of this it is difficult to predict variations in the apparent ionization constant of boric acid in solutions of different ionic strengths.   We found that increase in the borate concentration of the solution above $M/9$ caused appreciable variation in pH even with a constant buffer ratio.   Consequently we used $M/9$ borate solutions, although heavier buffering would otherwise have been desirable.

The final hemoglobin borate-buffered solutions were 7 per cent, or slightly more than 0.001 M in hemoglobin and $M/9$ in borate. The borate solutions were prepared in accordance with the method of Clark (6).   To a solution of 0.667 M boric acid and 0.667 M NaCl we added enough 0.667 N NaOH to adjust the hydrogen ion activity of the final buffered hemoglobin solution to the desired point.   In the case of these more alkaline solutions, the error due to the addition of incorrect amounts of alkali to the hemoglobin is more serious than is the pH shift following oxygenation.   We have, however, made calculations which indicate that the error in the former case probably does not exceed 0.02 in pH.   The change following oxygenation is limited because of the fact that at points more alkaline than pH 8.00 most of the hemoglobin, whether oxygenated or reduced, probably exists as salt.   Consequently the change in the value of the ionization constants has little effect on the pH.   At pH 8.88 we have calculated that the shift is about 0.01 in pH.

We have further controlled the accuracy of the pH estimations by determining the hydrogen ion activities of the buffered solutions of Preparations 28 and 29 electrometrically.   The measurements were made on solutions which had previously been reduced by repeated evacuation and equilibration with oxygen-free hydrogen. No deviation exceeding 0.01 in pH was detected between observed and calculated values.   This indicated that except for changing pH due to oxygenation, our oxygen dissociation curves are within the limits of experimental error, indeed isohydrionic.

Methods of Equilibration and Sampling.

Samples of buffered hemoglobin were equilibrated with analyzed gas mixtures and sampled by a method similar to that described by Austin *et al.* (7) as their first saturation method.

Suitable mixtures of oxygen and nitrogen were made each day and stored in a carboy over water acidulated with sulfuric acid. They were then analyzed with a Henderson-Haldane apparatus.

In order to equilibrate the buffered hemoglobin solutions with the gas mixtures, 10 to 15 cc. of solution were placed in a blood sampling tube of the usual design, and connected with a tonometer by means of rubber tubing.   Following evacuation the tonometer was filled with gas, slightly in excess of atmospheric pressure, and the hemoglobin solution introduced by appropriate manipulation of stop-cocks and the leveling bulb.   Subsequently the tonometer was rotated in a water bath at 25° ±0.02°.   After a few seconds, the gas was brought to atmospheric pressure by quickly opening and closing the tonometer stop-cock without removing the tonometer from the water bath.   After 5 minutes rotation, the tonometer was removed from the water bath and connected with the sampling tube into which the hemoglobin solution was next drawn.   Again the tonometer was evacuated and filled with gas mixture and, as before, the hemoglobin was reintroduced into the tonometer, which was then rotated in the water bath.   This procedure was repeated in all four times, except at low oxygen pressures, when five periods of rotation were employed.

As a result of a set of analyses performed after each period of rotation, we feel confident that equilibrium was actually reached at the end of this process. In order to be certain that the gas mixture in the tonometer was in fact identical with that in the carboy, we washed all connections with the mixture before connection was established either with the tonometer or the sampling tube.

After the completion of equilibration, the solution was returned to the sampling tube, and the tonometer removed. A pipette with stop-cock similar to those described by Van Slyke was substituted for the tonometer, and both pipette and connecting tube washed out with the gas mixture. The pipette was then filled and its contents rapidly transferred to a Van Slyke blood gas apparatus.

Following the removal of a sample for analysis, the remainder of the hemoglobin solution was returned to the tonometer and saturated with air at room temperature. This was done by washing out the tonometer frequently with moist air and shaking vigorously for at least 3 minutes. We believe that this constitutes an important step in the procedure, since large errors in the estimation of percentage saturation due to the formation of inactive hemoglobin during the process of equilibration, and resulting errors in the estimation of active hemoglobin, were thereby avoided. These errors are particularly significant on the acid side of the isoelectric point. A further advantage of this procedure is that errors due to changes in the concentration of hemoglobin, resulting from evaporation during manipulation, are avoided by this procedure.

Analysis for Oxygen Content.

The determination of the oxygen content of each sample was made with the Van Slyke constant volume blood gas apparatus, with carefully evacuated reagents. We believe that these analyses were accurate to about 0.05 volume per cent and that the combined errors of gas analysis, equilibration, sampling, and blood gas analysis rarely exceeded ±0.1 volume per cent, or, in general with the solutions here used, about 1 per cent saturation.

Oxygen was absorbed in the pipette by means of an alkaline solution of sodium hydrosulfite and anthraquinone sulfonate made up as described by Fieser (8). The stop-cock of the burette was

scrupulously cleansed and greased after each determination. Because of the necessity for making measurements of the oxygen content and capacity simultaneously, it was impossible, with the two burettes available, to make these measurements in duplicate. We believe that our analytical results are accurate to about 0.05 volume per cent, or in the case of solutions of about 0.001 M hemoglobin, to rather better than 1 per cent saturation.

Results.

We have collected the experimental data in Table I.   Columns 1 to 4 refer to the hydrogen ion activity and ionic strength, the number of preparation, the number of experiment, and oxygen tension, respectively.   In Column 5 appears the figure for the total oxygen contained in the sample expressed as volumes per cent.   This value is in fact the sum of two quantities, free and chemically bound oxygen.   The former quantity may itself, possibly, consist of two parts, an amount of oxygen in physical solution, and a fraction considered as "absorbed," as suggested by Conant (9).   At the present time there is no positive evidence for the existence of the last mentioned fraction.   We have, therefore, disregarded this possibility.

In order to estimate the solubility of oxygen in the salt solutions used, we have employed the theory of Debye and Hückel. According to this theory, gases, as non-electrolytes should be subject only to "salting out" effects.   Therefore, the first term of the Debye-Hückel equation drops out, leaving an equation of the form $-\log \dfrac{S}{S_0} = K_s\mu$ in which K_s is a proportionality constant characteristic of the salt used, S and S_0 the solubilities in the solvent and pure water respectively, and μ the ionic strength.   This has been discussed by Scatchard (10).   From a consideration of published data for the solubility of oxygen in salt solutions (11) it appears that this law holds.   Unfortunately there exist no data for phosphate solutions.[7]   We have, therefore,

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[7] It would, of course, be desirable to have data bearing directly on our problem.   Unfortunately these do not exist and it is extremely difficult to secure such data since relatively large changes in the electrolyte content of solutions alter the solubility coefficient to an extent which is not large compared to the experimental error.

TABLE I.

The results are expressed in volumes per cent. pO_2 in mm.

pa_H	Hb Preparation No.	Experiment No.	pO_2	O_2 content.	O_2 capacity.	O_2 combined.	O_2 combined, 100 per cent saturated.	Per cent saturation.
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
8.88	27	4	3.5	7.69	9.91	7.68	9.38	81.9
		12	4.7	8.42	9.88	8.40	9.36	89.7
		1	6.0	9.15	10.38	9.13	9.85	92.8
		7	10.8	9.10	9.97	9.06	9.44	96.0
		9	31.0	9.39	9.98	9.29	9.45	98.3
		14	62.9	9.59	9.87	9.38	9.34	100.4
8.40	27	6	3.5	7.22	10.20	7.21	9.67	74.6
		13	4.7	8.20	10.10	8.18	9.58	85.4
		2	6.0	8.70	9.93	8.68	9.40	92.3
7.95 $\mu = 0.48$	27	5	3.5	4.28	9.83	4.27	9.33	45.8
		3	6.0	7.59	9.72	7.57	9.23	82.0
		17	7.2	7.83	9.70	7.81	9.21	84.8
		8	10.8	8.74	9.98	8.71	9.48	91.9
		15	13.0	8.81	9.79	8.77	9.30	94.3
		10	31.0	9.46	10.16	9.36	9.66	96.9
7.65 $\mu = 0.46$	26	11	4.7	2.41	8.78	2.40	8.29	29.0
	28	19	8.1	5.72	9.04	5.69	8.54	66.6
	28	15	9.9	6.95	9.27	6.92	8.77	78.9
	28	8	18.8	8.13	8.96	8.07	8.47	95.3
7.38 $\mu = 0.453$	26	9	3.7	1.08	8.95	1.07	8.46	12.6
	23	9	5.5	1.80	8.60	1.78	8.11	21.9
	23	11	7.7	3.44	8.38	3.42	7.89	43.3
	28	20	8.1	3.62	9.00	3.59	8.50	42.3
	28	14	9.9	4.83	9.10	4.80	8.60	55.8
	23	5	11.2	5.56	8.41	5.53	7.91	70.0
	21	12	12.2	5.89	8.90	5.85	8.40	69.7
	28	13	14.8	6.99	9.12	6.94	8.62	80.5
	28	7	18.8	7.40	8.76	7.34	8.27	88.8
	22	11	27.0	5.46	6.16	5.38	5.67	94.9
	25	14	28.6	7.75	8.51	7.66	8.02	95.6
7.00* $\mu = 0.394$	28	1	7.2	1.37	8.81	1.35	8.32	16.2
	27	16	13.0	3.53	9.30	3.49	8.81	39.6
	28	10	15.8	4.67	8.84	4.62	8.34	55.4
	28	9	22.7	6.96	8.86	6.89	8.37	82.4
	28	3	26.5	7.44	8.82	7.36	8.33	88.3

TABLE I—*Continued.*

pa_H	Hb Preparation No.	Experiment No.	pO_2	O_2 content.	O_2 capacity.	O_2 combined.	O_2 combined, 100 per cent saturated.	Per cent saturation.
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
6.77	24	7	4.8	0.87	12.00	0.85	11.49	7.4
$\mu = 0.344$	24	6	5.8	1.04	11.81	1.02	11.31	9.0
	24	5	9.6	2.06	11.44	2.03	10.94	18.6
	21	9	11.9	2.50	8.90	2.46	8.40	29.3
	24	4	14.9	4.31	11.63	4.26	11.11	38.3
	24	3	21.2	7.08	11.44	7.01	10.92	64.2
	22	15	22.3	4.09	6.22	4.02	5.71	70.4
	22	12	27.0	4.83	6.24	4.74	5.73	82.7
	22	6	27.6	7.40	9.42	7.31	8.90	82.2
	24	2	30 0	9.42	11.50	9.32	10.98	84.9
	22	5	41.6	8.57	9.33	8.44	8.82	95.7
	24	1	43.3	10.43	11.32	10.29	10.81	95.3
6.60*	23	3	11.2	1.85	8.29	1.81	7.78	23.3
$\mu = 0.314$	25	3	43.0	8.26	9.49	8.12	8.94	90.8
6.55	28	12	15.8	2.82	8.79	2.77	8.28	33.5
$\mu = 0.299$	29	5	20.9	4.71	8.13	4.64	7.62	61.0
	28	5	26.5	5.89	8.77	5.81	8.27	70.3
	25	13	28.6	5.85	8.31	5.76	7.81	73.8
	25	11	32.6	6.75	8.91	6.65	8.40	79.2
6.50	25	9	21.3	4.84	8.72	4.77	8.21	58.1
$\mu = 0.288$	25	8	31.5	6.57	8.75	6.47	8.24	78.5
	25	6	36.8	7.56	9.13	7.44	8.61	85.3
	25	2	43.0	7.70	9.03	7.56	8.52	88.8
	25	5	46.9	7.98	8.87	7.83	8.36	93.7
6.40*	23	8	5.5	0.69	8.51	0.67	7.99	8.4
$\mu = 0.274$	23	2	11.2	1.65	8.20	1.61	7.67	21.0
	21	10	11.9	2.23	8.90	2.19	8.38	26.1
	28	11	15.8	2.61	8.64	2.56	8.11	31.5
	29	1	21.0	4.87	8.45	4.80	7.92	60.6
	22	19	23.2	3.11	5.00	3.03	4.48	67.6
	22	8	27.4	4.39	6.05	4.30	5.52	77.9
	22	9	27.4	4.33	6.17	4.24	5.64	75.2
	25	7	36.8	7.28	8.64	7.16	8.11	88.3
	22	4	41.6	8.14	9.33	8.00	8.80	90.9
	25	1	43.0	7.82	8.99	7.68	8.46	90.8
	25	4	46.9	7.94	8.80	7.79	8.27	94.2

TABLE I—*Concluded.*

pa_H (1)	Hb Preparation No. (2)	Experiment No. (3)	pO_2 (4)	O_2 content. (5)	O_2 capacity. (6)	O_2 combined. (7)	O_2 combined, 100 per cent saturated. (8)	Per cent saturation. (9)
6.18	28	2	7.2	0.96	8.78	0.94	8.26	11.4
$\mu = 0.232$	28	4	26.5	5.65	8.72	5.56	8.20	67.8
	28	6	26.5	5.85	8.62	5.76	8.10	71.1
	25	10	32.6	6.87	8.54	6.76	8.02	84.3
	25	12	34.0	6.88	8.37	6.77	7.85	86.2
5.8* $\mu = 0.207$	22	13	27.2	4.47	5.97	4.35	5.44	80.0
4.9	21	11	11.9	2.94	8.90	2.90	8.38	34.6
$\mu = 0.188$	29	6	20.9	5.06	8.05	4.99	7.53	66.4
	29	3	25.6	5.69	7.62	5.61	7.10	79.0
4.5* $\mu = 0.184$	29	2	21.0	5.38	7.25	5.31	6.72	79.0

* Electrometric measurement not made. pa_H interpolated from E.M.F. curve for phosphate-Hb system.

used the value for sodium chloride, $K_s = -0.14$.   This is justified since variations in K_s are not very great from salt to salt and also since the data for gas solubilities in electrolyte solutions are themselves so unsatisfactory that the value chosen accords within the experimental error with that obtained from the data for various salt solutions.

In addition to the salting out effect produced by the phosphates, we have also considered the effect of hemoglobin itself in lowering gas solubility.   The solubility may be assumed to be proportional to the volume of phosphate solution present per cc. of buffered hemoglobin solution.   This proportionality factor should be equal to the density of the buffered hemoglobin solution less the weight of hemoglobin in 1 cc. divided by the density of the phosphate solution.   No data exist for the densities of such buffered solutions, nor did it appear of sufficient importance to secure the data ourselves.   We have, therefore, estimated this factor as being approximately equal to 0.94.   This estimate is probably accurate to about ±2 per cent.

In Table II are given values for the solubility correction per mm. of oxygen pressure in terms of the ordinary solubility coefficient α, together with figures indicating their derivation. In order to convert these values to volumes per cent one must multiply by 100.

In Column 6 of Table I appears the value for oxygen capacity; that is, the total amount of oxygen per cc. of solution after saturation with air. In Columns 7 and 8 appear the values for the

TABLE II.

$$\text{Log } \frac{S}{S_0} \quad - \; 0.14$$

μ	$\text{Log } \dfrac{S}{S_0}$	$\dfrac{S}{S_0}$	$\dfrac{S}{S_0} \times \alpha = \alpha_1$	$\dfrac{\alpha_1}{760}$	$0.94 \times \dfrac{\alpha_1}{760}$
0.478	−0.066	0.859	0.025		
0.464	−0.065	0.860	0.025	0.000033	0.000031
0.453	−0.063	0.865	0.025		
0.394	−0.055	0.881	0.025		
0.344	−0.048	0.895	0.026		
0.314	−0.044	0.904	0.026	0.000034	0.000032
0.299	−0.042	0.908	0.026		
0.274	−0.038	0.916	0.027		
0.232	−0.032	0.929	0.027		
0.207	−0.029	0.935	0.027	0.000036	0.000033
0.188	−0.026	0.940	0.027		
0.184	−0.026	0.940	0.027		

combined oxygen after equilibration with the indicated oxygen tension and of another aliquot equilibrated with air respectively. Column 9 is self-explanatory.

The data are graphically represented in Figs. 1 and 2.

The data here presented are, we believe, in general accurate to about 1 per cent saturation, but in the case of the more acid solutions, changes in the hemoglobin occurred so rapidly that such data possess only qualitative significance.

Fig. 2.

Oxygen Pressure in Mm.

Percent Saturation

Fig. 1.

Oxygen Pressure in Mm.

Percent Saturation

▼ = 6.50
● = 6.55
□ = 6.60
+ = 6.77
● = 7.00
▽ = 7.56
○ = 7.65
+ = 7.35
× = 8.40
● = 888
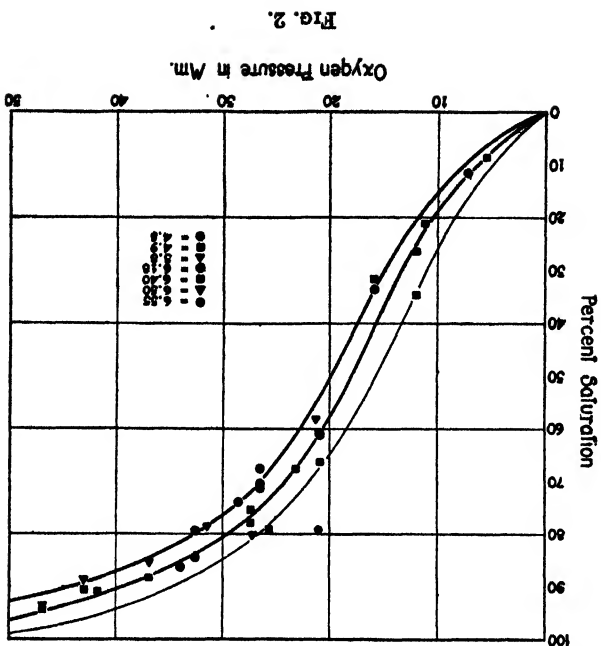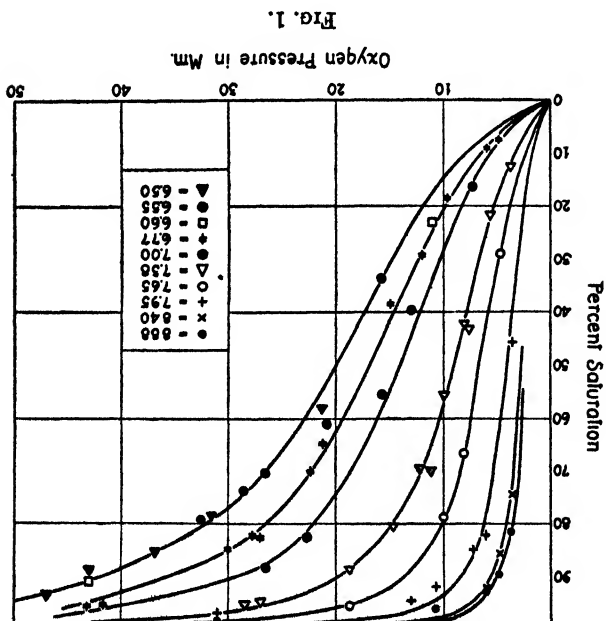
DISCUSSION.

The classical interpretation of the equilibrium between oxygen and hemoglobin has been based on the hypothesis that they combine according to an equation of the form $Hb_n + nO_2 \rightleftharpoons Hb_nO_{2n}$. In this case a simple equilibrium constant may be written

$$K \quad \frac{{}^a Hb_nO_{2n}}{{}^a Hb_n \cdot {}^a O_2{}^n}$$

This should hold provided that the proper values are used for the activities. If we replace activities by stoichiometric concentrations, we must write

$$K' = \frac{[Hb_n \, O_{2n}]}{[Hb_n] \, [O_2]^n} \tag{1}$$

where $K' = K \dfrac{\gamma'}{\gamma_1}$ and γ_1 and γ' are activity coefficients referring to oxyhemoglobin and reduced hemoglobin, respectively.

The simple theory has previously been proved faulty. Further proof is given by the curves in Fig. 3, in which $\log R = \log \dfrac{Hb}{HbO_2}$ has been plotted against $\log pO_2$. In order to conform to the theory, these curves should represent a family of straight lines —a consideration already discussed fully by one of us (12) and by Brown and Hill (13). Only at pH 7.38 does this hypothesis nearly fit, and here the value for n is approximately 2.6, a value closely comparable to the value, 2.4, found at a similar pH in human blood. The fact that the assumption of integral values of n in Equation 1 does not accord with the facts, is shown in Fig. 4 in which oxygen dissociation curves constructed according to this hypothesis are compared with the data obtained at pH 6.77, and with a curve drawn according to a theory subsequently discussed.

We are, then, compelled to assume either (1) that the reduced-oxyhemoglobin activity coefficient ratio varies with oxygenation, or (2) that the correction for dissolved oxygen should itself be corrected on account of the presence of "adsorbed" oxygen, or (3) that the theory does not accord with the facts. As far as the

first alternative is concerned, one might, on account of the fact—clearly shown by L. J. Henderson (14)—that oxyhemoglobin is a stronger acid than reduced hemoglobin, expect variation in the
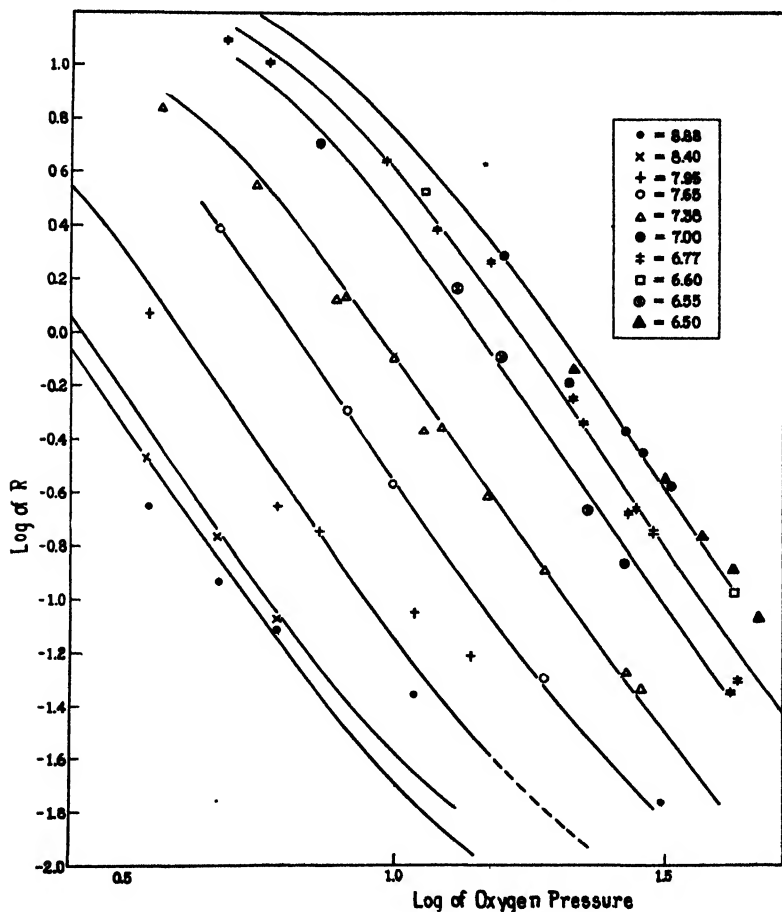
Fig. 3.

activity coefficient ratio in unbuffered hemoglobin solutions. But in the buffered solutions used by us, we are unable to describe a mechanism which would vary the ratio in such a manner as to give a single isohydrionic curve, to say nothing of the family of

curves in Fig. 3.   The second alternative has as yet no experimental support[8] and seems unlikely.   We are, consequently, compelled to adopt the third alternative.

Henderson (15) has developed an empirical equation of the form $y = a - bx - cx^2$, in which $y = \log pO_2$, $x = \log R$, b and c are constants, while a is a constant which is a linear function of $\log pCO_2$ having the value of $\log pO_2$ at 50 per cent saturation. We have been unable to fit all our curves by an equation of this
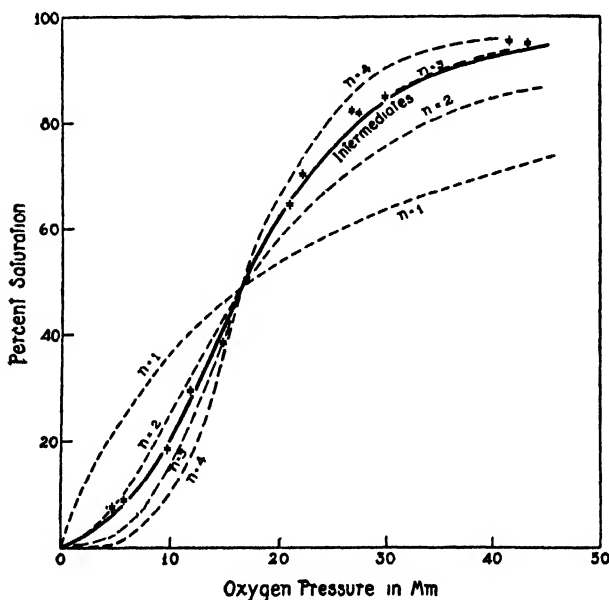
FIG. 4.

type.   Further, it presents no apparent theoretical justification.

We have also developed a simple empirical expression which may be written:

$$K = \frac{[Hb][pO_2]}{[HbO_2]} = 0.5 + \frac{[H^+]}{0.013(7.4 \times 10^{-8} + [H^+])}(\log \% \, HbO_2 - 2.015) \quad (2)$$

in which the symbols have the same meaning as in Equation 1.

[8] J. B. Conant—personal communication—has not been able to secure any experimental evidence substantiating this view.

In this equation the equilibrium constant is a linear function of the log of the mol fraction of oxyhemoglobin. This expression accords with the data at most of the pH values used in our experi-
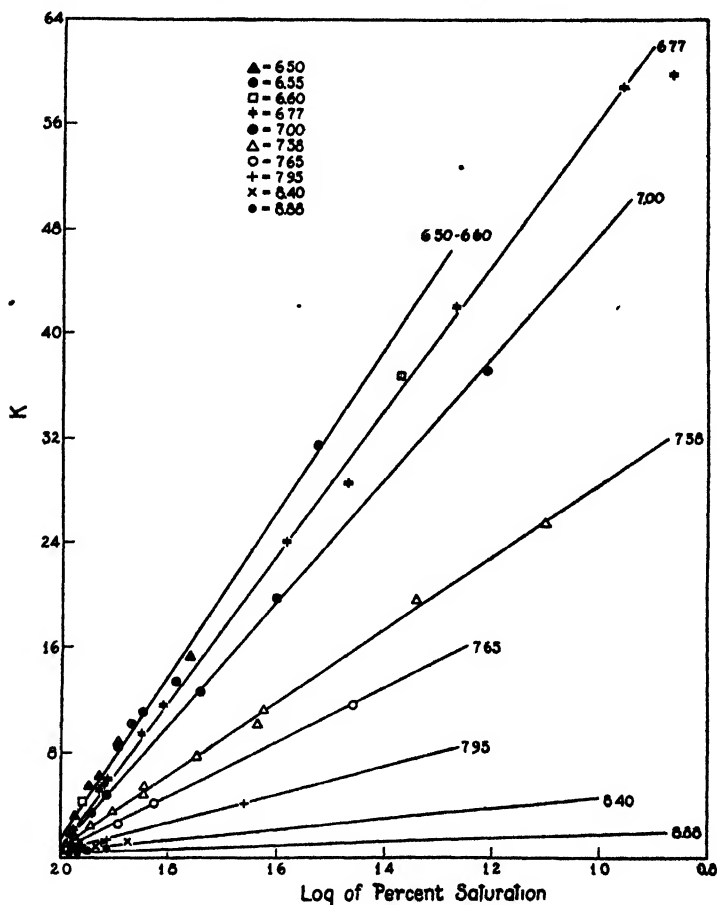
Fig 5.

ments. This is shown in Fig. 5, in which we have plotted K against log per cent HbO_2. Only at pH 6.55 does the deviation from the data become significant. This expression moreover demon-

strates the observed fact that oxygenation increases the affinity of hemoglobin for oxygen, since the value of K decreases as oxygenation at constant pH progresses.   Further, it indicates the fact that increasing pH increases the affinity of hemoglobin for oxygen by a decreasing value of K.   We have thus far been unable to attach any theoretical significance to this expression.   It may, nevertheless, serve as a useful tool in further investigation.

The experimental data at any pH may be described in terms of equations in which hemoglobin appears as a mixture of forms each of which reacts according to Equation 1, where n is an integer. This form of expression was suggested by Redfield (16) who found that the oxygen dissociation curve of the hemocyanin occurring in *Limulus polyphemus* could be satisfactorily described in these terms.   One may assume a mixture of equal parts of hemoglobin such that $n = 2$ and $n = 4$, or a mixture such that there is present one-fifth in the form in which $n = 1$, and four-fifths in which $n = 4$.   Accordingly equations may be written

$$\text{Per cent saturation} = \frac{50K_2\,[O_2]^2}{1 + K_2\,[O_2]^2} + \frac{50K_4\,[O_2]^4}{1 + K_4\,[O_2]^4} \quad \text{or}$$

$$\text{Per cent saturation} = \frac{20K_1\,[O_2]}{1 + K_1\,[O_2]} + \frac{80\,K_4\,[O_2]^4}{1 + K_4\,[O_2]^4}$$

in which K_1, K_2, and K_4 are the equilibrium constants of Equation 1 when n is 1, 2, and 4, respectively.   All the curves may be fitted on either assumption.[9]   Further, these constants vary systematically with pH.   In this form of expression only two constants are needed to fit a single isohydrionic curve.   But at present the

[9] Although we have not presented curves drawn according to this hypothesis, they may be constructed by means of the following constants when pO_2 is used as a measure of O_2 concentration:

	pH	6.55	6.77	7.00	7.38	7.65	7.95	8.40	8.88
$n = 2$	$\sqrt[2]{K_2}$	15.8	13.8	11.5	7.9	5.8	4.3	3.2	2.5
$n = 4$	$\sqrt[4]{K_4}$	22.6	19.4	15.5	9.5	6.0	3.5	1.7	0.89
$n = 1$	K_1	8.4	7.5	6.5	4.9	4.0	3.2	2.9	2.5
$n = 4$	$\sqrt[4]{K_4}$	21.8	18.8	15.2	9.7	6.5	4.2	2.5	1.9

The values given above vary with pH if pK is assumed to be 7.0.

molecular weight of this protein, 66,800, does not accord with this assumption, at least in its explicit form.

As another alternative we may use the theory of intermediate compound formation suggested by Adair (17). This theory follows rather naturally from the observed molecular weight of hemoglobin (66,800), which together with the established iron-oxygen ratio (18), implies that the 4 iron atoms in the hemoglobin molecule are each through the radicals they represent capable of combining with 1 oxygen molecule.   .

We know nothing of the spatial arrangements of these groups. But no matter what their spatial relations may be, we may assume that each group combines, when fully saturated, with 1 oxygen molecule. We have, therefore, to deal with the series of compounds Hb_4, Hb_4O_2, Hb_4O_4, Hb_4O_6, Hb_4O_8, in which Hb_4 represents the presence of 4 atoms of iron, and may write:

$$Hb_4 + O_2 = Hb_4O_2, \quad K_1 = \frac{a_{Hb_4O_2}}{a_{Hb_4} \cdot a_{O_2}}, \quad a_{Hb_4O_2} = K_1 \, a_{Hb_4} \cdot a_{O_2}$$

$$Hb_4O_2 + O_2 = Hb_4O_4, \quad K_2 = \frac{a_{Hb_4O_4}}{a_{Hb_4O_2} \cdot a_{O_2}}, \quad a_{Hb_4O_4} = K_1 \, K_2 \, a_{Hb_4} \cdot a_{O_2}^2$$

$$Hb_4O_4 + O_2 = Hb_4O_6, \quad K_3 = \frac{a_{Hb_4O_6}}{a_{Hb_4O_4} \cdot a_{O_2}}, \quad a_{Hb_4O_6} = K_1 \, K_2 \, K_3 \, a_{Hb_4} \cdot a_{O_2}^3$$

$$Hb_4O_6 + O_2 = Hb_4O_8, \quad K_4 = \frac{a_{Hb_4O_8}}{a_{Hb_4O_6} \cdot a_{O_2}}, \quad a_{Hb_4O_8} = K_1 \, K_2 \, K_3 \, K_4 \, a_{Hb_4} \cdot a_{O_2}^4$$

The symbols used here have the usual significance: a_{Hb_4}, $a_{Hb_4O_2}$, $a_{Hb_4O_4}$, $a_{Hb_4O_6}$, $a_{Hb_4O_8}$, a_{O_2}, refer to the activities of reduced, monooxy-, dioxy-, trioxy-, and tetraoxyhemoglobin and oxygen respectively. These are the substances postulated by the theory of intermediate compound formation. Substituting the product of an activity coefficient and the stoichiometric concentration for each of the terms involving hemoglobin and $[O_2]$ for the pressure of oxygen, we can write:

$$[Hb_4O_2] = K_1'\gamma' \, [Hb_4] \, [O_2]$$
$$[Hb_4O_4] = K_1'K_2'\gamma' \, [Hb_4] \, [O_2]^2$$
$$[Hb_4O_6] = K_1'K_2'K_3'\gamma' \, [Hb_4] \, [O_2]^3$$
$$[Hb_4O_8] = K_1'K_2'K_3'K_4'\gamma' \, [Hb_4] \, [O_2]^4$$

where $K_1' = \dfrac{K_1}{\gamma_1}$, $K_2' = \dfrac{K_2}{\gamma_2}$, $K_3' = \dfrac{K_3}{\gamma_3}$, $K_4' = \dfrac{K_4}{\gamma_4}$, and γ', γ_1, γ_2, γ_3, and γ_4, the activity coefficients for $[Hb_4]$, $[Hb_4O_2]$, $[Hb_4O_4]$, $[Hb_4O_6]$, and $[Hb_4O_8]$ respectively.

The concentration of combined oxygen may now be written:

$$Hb_4\gamma' \, (K_1' \, [O_2] + 2 \, K_1'K_2' \, [O_2]^2 + 3 \, K_1'K_2'K_3' \, [O_2]^3 + 4 \, K_1'K_2'K_3'K_4' \, [O_2]^4)$$

and the total concentration of protein

$$Hb_4\gamma' \, (1 + K_1' \, [O_2] + K_1'K_2' \, [O_2]^2 + K_1'K_2'K_3' \, [O_2]^3 + K_1'K_2'K_3'K_4' \, [O_2]^4)$$

Now since 4 oxygen atoms combine with hemoglobin when oxygenation is complete, we may write:

$$\text{Per cent saturation} = \frac{\frac{1}{4} \, [\text{concentration of combined } O_2]}{\text{total Hb concentration}} \times 100$$

$$\frac{100 \, \gamma' \, [Hb_4] \, (K_1' \, [O_2] + 2 \, K_1'K_2' \, [O_2]^2 + 3 \, K_1'K_2'K_3' \, [O_2]^3 + 4 \, K_1'K_2'K_3'K_4' \, [O_2]^4)}{4 \, \gamma' \, [Hb_4] \, (1 + K_1' \, [O_2] + K_1'K_2' \, [O_2]^2 + K_1'K_2'K_3' \, [O_2]^3 + K_1'K_2'K_3'K_4' \, [O_2]^4)} \quad (3)$$

If all the oxygen-binding groups have the same affinity for oxygen, the saturation of any one group has no chemical effect on any of the others—as might be the case if they were widely separated in space—and the order of saturation is governed by probability, then, as Adair has shown,

$$K_2' = \frac{3}{8} \, K_1', \; K_3' = \frac{1}{6} \, K_1', \; K_4' = \frac{1}{16} \, K_1'$$

and the expression reduces to

$$\text{Per cent saturation} = y \qquad \frac{0.25 \, K_1'}{1 + 0.25 \, K_1'}$$

a transformation of Equation 1. This is known not to hold. Adams (19) and later Simms (20) have developed a similar treatment for the ionization of polyvalent organic acids.

Adair (17) has been able rather successfully to express his own data, obtained at 37° on human hemoglobin, in terms of the equation:

$$y = \frac{0.25 \, K \, [O_2] + 0.25 \, (K \, [O_2])^2 + 0.25 \, (K \, [O_2])^3 + (K \, [O_2])^4}{1 + (K \, [O_2]) + 0.5 \, (K \, [O_2])^2 + 0.333 \, (K \, [O_2])^3 + (K \, [O_2])^4} \quad (4)$$

a form which is the result of substituting the following values in Equation 3: $K = K_1'$, $K_2' = 0.5\ K_1'$, $K_3' = 0.667\ K_1'$, $K_4' = 3\ K_1'$. These values do not, however, satisfy our data obtained on horse hemoglobin at 25°. But if we substitute the values $K_2' = 0.32\ K_1'$, $K_3' = 0.8\ K_1'$, $K_4' = 10.5\ K_1'$ in Equation 3, we obtain Equation 5.[10]

$$y = \frac{0.25\,K_1'[O_2] + 0.50 \times 0.32\,(K_1'[O_2])^2 + 0.75 \times 0.256\,(K_1'[O_2])^3 + 2.7\,(K_1'[O_2])^4}{1 + K_1'[O_2] + 0.32\,(K_1'[O_2])^2 + 0.256\,(K_1'[O_2])^4 + 2.7\,(K_1'[O_2])^4} \quad (5)$$

a form in which it is possible satisfactorily to describe our data. We have, moreover, constructed the curves in Figs. 1 and 3 according to this equation. It has thus been possible, with four constants bearing a definite ratio to each other at all pH values from 6.55 to 8.88, to describe the equilibrium between oxygen and hemoglobin at any pH within this range.

The agreement between the theoretical curves and the data is rather gratifying, especially when the deviations are critically considered. Turning to Fig. 1, we note that agreement between data and theory is very good up to pH 7.95, a point at which we have previously noted the inefficiency of phosphate buffers. Furthermore, the discrepancy is serious only above 90 per cent saturation, and quantitatively about what one would expect for a shift of 0.05 in pH. That this may be the correct explanation is suggested by the rather better agreement at pH 8.40 and 8.88, where borate buffers were used. Turning now to Fig. 3, we again note excellent agreement up to pH 7.95. In the case of the curve for pH 7.95, both the magnitude and the direction of the deviation are what one would expect on the ground of inefficient phosphate buffering, with consequent shift in pH. In the case of the curves for pH 8.40 and 8.88, agreement between theory and data is reasonably good until the value for log R assumes a negative value greater than -1.2. This is the point where small errors in per cent saturation result in large errors in log R.

None the less, it is to be remembered that we have used four constants and that—as our empirical equations have shown—any equation that will describe one curve will describe the rest.

We wish also to call attention to the fact that we have made no correction for possible variation of the activity coefficients of the

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[10] It can be shown that positive roots of this equation are uniquely determined.

various ionic species of hemoglobin, of which there must be at least five present. We know no means of estimating this factor at present and the correction is in part implicitly assumed in the various K' factors. But we are assuming that the thermodynamic environment is constant for the entire family of curves, an assumption that is certainly incorrect since μ varies from 0.288 to 0.464.

Again, these results may be applicable only to horse hemoglobin prepared according to the method described. It is not proper to extrapolate these results to whole blood without further study.

But in spite of our reservations, if one makes the single assumption of the existence of intermediates, the molecular weight of hemoglobin (66,800), together with the iron-oxygen ratio, necessitates the general equations developed above. It must be admitted, however, that there is at present no direct experimental evidence in favor of this assumption.

We can now turn to the effect of changes in hydrogen ion activity on the equilibrium studied. Even from Figs. 1 and 2 it is apparent that the affinity of hemoglobin solutions for oxygen passes through a minimum somewhere between pH 6 and 7. This fact was first suggested by the work of Rona and Ylppö (21). The phenomenon is more clearly shown in Fig. 6, in which we have plotted the oxygen pressure sufficient to produce 50 per cent saturation against pH.

Fig. 6 is also useful in showing that the constant a in Henderson's equation is not a linear function of pH, but must be explained on some other basis. Inspection of this figure also strengthens the conviction, previously expressed by Adolph and Ferry (22), that oxygen binding is in some way dependent on the ionization, and therefore, on the acid or basic dissociation constants of hemoglobin. The curve in this figure is strongly reminiscent of a titration curve, and suggests that, while acid dissociation constants may be chiefly effective on the alkaline side of the point of minimum oxygen binding, basic constants may predominate at more acid reactions. We are unable to analyze this curve further.

Let us next consider the first equation of the series upon which the theory of intermediates is based, $K_1' = \dfrac{[\mathrm{Hb_4\, O_2}]}{\gamma'\,[\mathrm{Hb_4}]\,[\mathrm{O_2}]}$. The constants for the equilibrium between oxygen and the unionized free acid and its ionized salt are probably different, so that we may write, after Henderson:

$$K_a = \frac{a_{H_2Hb_4O_2}}{a_{H_2Hb_4} \cdot a_{O_2}}, \ [H_2Hb_4O_2] = K_a' \ [H_2Hb_4] \ [O_2] \ \text{where} \ K_a' = K_a \frac{\gamma_{H_2Hb_4}}{\gamma_{H_2Hb_4O_2}}$$

$$K_s = \frac{a_{B_2Hb_4O_2}}{a_{B_2Hb_4} \cdot a_{O_2}}, \ [B_2Hb_4O_2] = K_s' \ [B_2Hb_4] \ [O_2] \ \text{where} \ K_s' = K_s \frac{\gamma_{B_2Hb_4}}{\gamma_{B_2Hb_4O_2}}$$

$$K_R = \frac{a_{B_2Hb_4} \cdot a_{H^{+2}}}{a_{H_2Hb_4}}, \ K_R' = \frac{[B_2Hb_4] \ [H^+]^2}{[H_2Hb_4]} \ \text{where} \ K' = \frac{K_R}{\gamma_{B_2Hb_4}}$$

if we assume that over the pH range studied the acid group affected by oxygenation behaves as though it were a dibasic acid H_2Hb_4 with salt B_2Hb_4.[11]   We should then write:

$$K_1' = \frac{[\text{total monooxyhemoglobin}]}{[\text{total reduced hemoglobin}] \ [O_2]} = \frac{(K_a' \ [H_2Hb_4] + K_s' \ [B_2Hb_4]) \ [O_2]}{([B_2Hb_4] + [H_2Hb_4]) \ [O_2]}$$

Substituting for H_2Hb_4 its equivalent, $\dfrac{B_2Hb_4 \ [H^+]^2}{K_R'}$, we may write:

$$K_1' = \frac{\dfrac{K_a' \ [B_2Hb_4] \ [H^+]^2}{K_R'} + K_s' \ [B_2Hb_4]}{[B_2Hb_4] + \dfrac{[B_2Hb_4] \ [H^+]^2}{K_R'}}$$

Simplifying

$$K_1' = \frac{K_a' \ [H^+]^2 + K_s' \ K_R'}{K_R' + [H^+]^2} \tag{6}$$

We can now solve for K_R' and write:

$$K_R' = \frac{[H^+]^2 \ [K_1' - K_a']}{[K_s' - K_1']} \tag{7}$$

If we now let $K_a' = 0.045$ and $K_s' = 0.345$ and $K_R' = 0.158 \ \times$

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[11] Redfield and Mason (23) have shown that upon the addition of HCl, *Limulus* hemocyanin is transformed from a colored to a colorless form, which does not combine with oxygen, according to a similar equation.   It is also to be noted that the change in solubility of carboxyhemoglobin in concentrated salt solutions over the range pH 6.6 to 7.4 may be described by assuming that it dissociates as a divalent acid (24).

10^{-7}, we may draw the curve shown in Fig. 7. The values for K_1' obtained from our data are:[12]

pH	8.88	8.40	7.95	7.65	7.38	7.00	6.77	6.55
K_1'	0.35	0.32	0.21	0.13	0.09	0.06	0.05	0.042
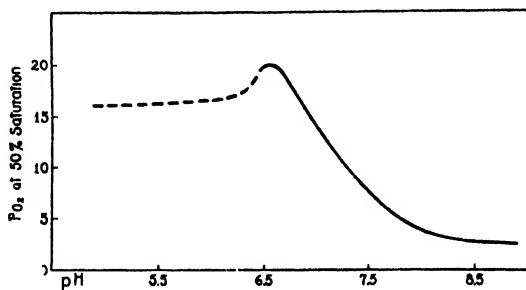
FIG. 6.
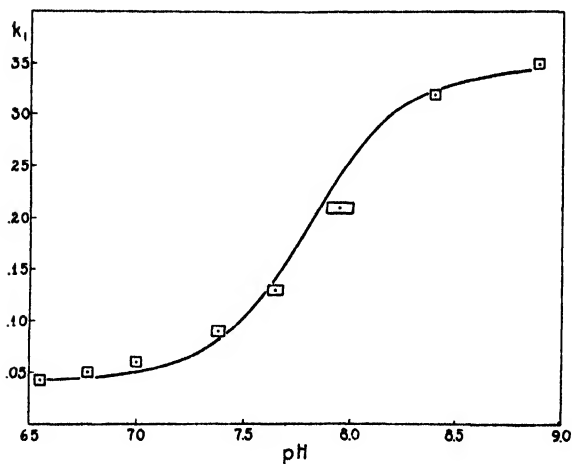
FIG. 7.

[12] The value for $pK_R' = 7.80$ differs from that found by Hastings (25) for reduced hemoglobin. Hastings' value of $pK_R' = 8.18$ obtained in solutions of $\mu = 0.30$, is, however, based on titration of all the acid groups. We have good reasons for believing that only a small number of these groups is involved in the oxygenation process. This has already been shown by Redfield and Mason (23) for hemocyanin.

These points are shown as rectangles on Fig. 7. The size of the rectangles indicates graphically the probable order of their accuracy. It is worth while noting again that the error is greatest at pH 7.95.

This procedure gives a theoretical basis for the variation of K_1' with pH and together with Equation 5 will explain the equilibrium between oxygen and solutions of purified horse hemoglobin over the range pH 6.55 to 8.88. The fact that only a few of the many acidic hydrogen atoms in hemoglobin. are intimately connected with the process of oxygenation supports the idea that hemoglobin may be a complex iron salt somewhat analogous to the complex cyanides (26).

CONCLUSIONS.

1. An improved method for the preparation of hemoglobin is described. This depends on repeated crystallization of oxyhemoglobin from a cold concentrated solution after complete oxygenation and adjustment of the pH to a point slightly more acid than pH 6.8.

2. Buffered solutions of hemoglobin of given pH were made by the addition of water, phosphate or borate buffers, together with enough alkali to change the pH of reduced hemoglobin to the same value. These solutions were equilibrated with known gas mixtures and analyzed by the methods of Van Slyke.

3. Oxygen dissociation curves were constructed from the data so obtained and show the familar S-shape and the decrease of percentage saturation with increasing acidity up to pH 6.55. At higher hydrogen ion activities the percentage saturation again rises. The affinity of hemoglobin solutions for oxygen passes through a minimum between pH 6 and 7, apparently at 6.55.

4. It is impossible to describe these data in terms of the simple mass law equation. More variables than have been commonly used must be employed.

5. The equilibrium between oxygen and hemoglobin solutions may be described by means of a simple empirical equation of the form

$$\frac{[Hb]\ [pO_2]}{[HbO_2]} = 0.5 \frac{[H^+]}{0.013\,(7.4 \times 10^{-8} + [H^+])}\ (\log \%\ HbO_2 - 2.015)$$

6. These experimental findings may also be expressed in terms of equations in which it is assumed that hemoglobin behaves as though it were a mixture of substances, each obeying the general form of the mass law equation where n is an integer.

7. These data are also described in terms of the theory of intermediate compound formation by the equation

Per cent saturation =

$$100\frac{0.25 K_1' O_2 + 0.5 \times 0.32 (K_1' [O_2])^2 + 0.75 \times 0.256 (K_1' [O_2])^3 + 2.7 (K_1' [O_2])^4}{1 + K' [O_2] + 0.32 (K' [O_2])^2 + 0.256 (K' [O_2])^3 + 2.7 (K' [O_2])^4}$$

8. If the theory of intermediate compound formation be assumed, the change in position of oxygen dissociation curves with changing pH, over the range 6.55 to 8.88, may be explained on the assumption that the salt and acid forms of hemoglobin have different affinities for oxygen.   A theoretical expression of the form

$$K_1' = \frac{K_R' K_s' + K_a' [\text{H}^+]^2}{[\text{H}^+]^2 + K_R'}$$

will explain the phenomenon.

9. Owing to the large number of assumptions involved, any theory that may at present be advanced must await further investigation for its verification.

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THE RELATIONS OF THE SERUM PROTEINS AND LIPIDS TO THE OSMOTIC PRESSURE.

By ELLA H. FISHBERG.

(*From the Chemical Laboratory of Beth Israel Hospital, New York.*)

The chemical composition of the blood of rabbits following re-peated heavy hemorrhages is in many respects similar to the con-dition of the blood of patients suffering from nephrosis. The con-stant removal of serum proteins, in the former case by actual loss of blood (Fishberg (1, 2)), in the latter by passage through the cells of the kidney into the urine, results in a disturbance of the mechanism for the maintenance of the colloid osmotic pressure of the blood. Following this loss of protein the blood shows a much increased lipid content.

Boggs and Morris (3) attributed the lipemia to the lowered oxidation caused by the loss of red blood corpuscles, while Sakai (4) and Horiuchi (5) thought that the lowering of the blood lipase might be the determining factor. Bloor (6) believes that nothing definite can be said as to the cause of this rise in the lipid content of the blood. We have made a quantitative study of (1) the re-lation between the colloid osmotic pressure and the concentration of the serum proteins; (2) the relation between the serum proteins and the lipids; (3) the relation between the colloid osmotic pres-sure, the proteins, and the lipids in an effort to throw some light on this problem.

1. Relation between Colloid Osmotic Pressure and Concentration of Serum Proteins.

Since the initial communication of Starling (7) it has been known that the serum proteins exert an osmotic pressure. The same investigator (8) showed later that a rough proportionality obtains between the colloid osmotic pressure and the concentration of the protein in the serum. It has been found that as the serum is

diluted, the colloid osmotic pressure falls more sharply than does the concentration of the serum proteins. Verney (9) believes that the explanation of this fact is to be found in the comparatively large molecular volume occupied by the colloidal particles so that the situation is analogous to a highly compressed gas. He finds that where p is the colloid osmotic pressure of the proteins, v the reciprocal of the protein concentration, and b a constant dependent on the particular condition, in analogy with Van der Waal's equation

$$p(v-b) = k$$

holds for concentrations down to 50 per cent of that of normal serum. Thus he found that the colloid omotic pressure of the serum proteins varies with their dilution within definite limits in the manner that would be expected of a non-ionized colloidal solution in which the colloidal particles occupy an effective volume as large as 50 per cent of the original.

It is known that the osmotic pressure of the serum per gm. of protein becomes lower, the less the actual concentration of the proteins in the serum. If we assume that the rate of change of the pressure with respect to the concentration of the protein is proportional to the actual osmotic pressure, we have the differential equation

$$\frac{dp}{dv} = ap$$

where p is the reciprocal of the colloid osmotic pressure and v is the reciprocal of the protein concentration.

Separating the variables and integrating we have

$$\int \frac{dp}{p} = \int a\,dv$$

$$\log p = av$$

$$p = e^{av}$$

where a is a constant depending on the total concentration of the proteins in the blood. Using this constant we find a striking agreement not only with our own experimental results obtained

TABLE I.

Relation of Protein Concentration to Osmotic Pressure.

Protein in 10 cc. serum.	Osmotic pressure.	v	p	as calculated $(p = e^{av})$.	as observed.
gm.	mm.				
0.721	353	1.39	2.83	1.04	1.05
0.639	301	1.58	3.35	1.19	1.21
0.577	283	1.72	3.53	1.29	1.26
0.505	219	1.98	4.57	1.49	1.52
0.432	169	2.31	5.93	1.73	1.78
0.361	106	2.78	8.42	2.09	2.13
0.289	75	3.27	13.46	2.60	2.60
0.216	29	4.64	35.1	3.48	3.57

TABLE II.

Relation of Protein Concentration and Osmotic Pressure.

Author.	Protein.	Reciprocal of protein concentration. v	Osmotic pressure.	Reciprocal of osmotic pressure. p	as observed.	as calculated.
	gm: per 10 cc.		mm.			
Verney.	0.6943	1.44	336	2.98	1.1	1.1
	0.6490	1.54	324	3.24	1.17	1.15
	0.6355	1.57	295	3.39	1.22	1.18
	0.6262	1.60	282	3.55	1.27	1.2
	0.458	2.18	171	5.85	1.63	1.72
	0.434	2.30	149	6.71	1.9	1.8
	0.377	2.66	133	7.52	2.02	1.99
	0.356	2.83	117	8.55	2.15	2.12
	0.341	2.93	115	8.69	2.16	2.20
	0.334	3.00	108	9.26	2.23	2.25
	0.274	3.65	66	15.20	2.73	2.74
Krogh.	0.828	1.21	385	2.60	0.96	0.91
	0.414	2.42	150	6.67	1.9	1.82
	0.276	3.62	80	12.50	2.53	2.61
Mayrs.	0.788	1.27	402	2.49	0.92	0.95
	0.709	1.41	348	2.87	1.05	1.05
	0.630	1.59	276	3.62	1.29	1.27
	0.551	1.81	247	4.05	1.4	1.36
	0.473	2.11	202	4.95	1.60	1.59
	0.394	2.54	157	6.37	1.83	1.90
	0.315	3.17	118	8.47	2.13	2.30
	0.236	4.24	78	12.80	2.65	3.0

both with human blood and the blood of rabbits, but also with that obtained by Mayrs (10), Krogh and Nakazawa (11), and Verney (cf. Tables I to III).

Technique.—Serum proteins were determined by a micro modification of methods already known (Howe (12)); but as the method is generally applicable and requires only 0.5 to 1 cc. of blood, it may be given here.

Determination of Total Protein.—0.5 cc. of blood serum is diluted to 25 cc. with physiological salt solution. 1 cc. of this diluted serum is heated in a Folin non-protein nitrogen tube with 1 cc. of

TABLE III.

Relation of Serum Protein Concentration to Osmotic Pressure in Rabbit.

Protein	Total protein	Osmotic pressure	v	p	ar cal·u-lated	ar ob-served
gm per 100 cc.	*per cent*	*mm.*				
6.71	100	290	3.46	1.49	1.19	1.24
5.37	80	226	4 43	1.86	1.48	1 49
4.47	66 7	164	6.11	2.24	1.79	1.81
3.36	50	80	11.2	2.98	2.38	1· 42
2.69	40	49	20.1	3.72	2.97	the
1.68	25	9	115	5 95	4.76	pro-
5.75	100	234	4.27	1.74	1.45	1 45
4.60	80	172	5.82	2.17	1.76	1.77
3.83	66 7	119	8.41	2.61	2.18	2.13
2.87	50	56 6	17.7	3.40	2.81	2.87
2.30	40	27	32	4.35	3.63	3.58

digestion mixture (100 cc. of concentrated sulfuric acid are added to 10 cc. of 10 per cent copper sulfate and the mixture poured into 100 cc. of distilled water) and a glass bead till the solution becomes colorless. This is cooled and diluted to the 50 cc. mark.

Determination of Albumin.—0.5 cc. of blood serum is dropped into 16.2 cc. of 22.5 per cent sodium sulfate solution at 37°. There is a precipitate of globulin formed at once. This is kept for an hour at 37° in an incubator and then filtered through a double layer of Schleicher and Schüll filter paper. If the solution does not come through crystal-clear the filtration must be repeated. 1 cc. of this mixture is digested in the same way as above.

Comparison.—0.1414 gm. of ammonium sulfate per liter is taken as a standard. 5 cc. of this solution are put in a non-protein nitrogen tube with 1 cc. of digestion mixture, and the whole diluted to 35 cc. 15 cc. of Nessler's dilute solution are added to each tube and the color compared in a Duboscq color-imeter. We have

$$6\,25\left(\frac{750\,S_s}{S_u} - \text{N-PN}\right) = \text{protein (mg. per 100 cc. blood)}$$

$$6\,25\left(\frac{450\,S_s}{S_u} - \text{N-PN}\right) = \text{albumin (mg. per 100 cc blood)}$$

where S_u is the reading of the unknown solution and S_s is the reading of the standard solution and N-PN the concentration of non-protein nitrogen in the blood.

Protein − albumin = globulin

The osmotic pressure is measured by a technique similar to that used by Loeb (13), but membranes of approximately 5.5 cc. capacity are made by revolving collodion (Eimer and Amend) 2 minutes in flat bottomed tubes (12 mm. in diameter and 55 mm. in length) and allowing them to dry 2 minutes. A 1-hole rubber stopper is then inserted and with a pipette the membrane is filled, leaving no air bubbles. A capillary manometer tube of 0.8 mm. bore is then dipped into the serum and inserted into the opening in the rubber stopper, care being taken that no air is admitted. The serum shoots up in the tube at once if this is carefully done. The membrane is then fastened to the stopper by a layer of ad-hesive plaster and the whole made air-tight by a layer of paraffin. This is then inserted into a beaker containing Ringer's solution. The readings are taken after 18 hours and a capillary correction made.

The blood is obtained from the shaved ear of the rabbit by making a simple incision of the ear vein and then allowing the blood to drip into a test-tube. It takes approximately an hour to obtain 30 cc. of blood.

2. Relation of Proteins and Lipids of Blood.

An average of 30 cc. of blood was removed from the rabbits' ears and after 6 to 8 days when the red blood cell count had fallen under 2,000,000, the blood became turbid, and 2 days later definitely milky.

TABLE IV.

Relation between Lipids and Protein in Blood Serum.

Date.	Weight.	Blood drawn.	Total blood drawn.	Red blood cells.	Total protein.	Albumin.	Globulin.	Albumin:glo-bulin.	Cholesterol.	Total lipids.
1927	*gm.*	*cc.*	*cc.*	*million*	*gm. per cent*	*gm. per cent*	*per cent*		*mg. per 100 cc.*	*mg. per 100 cc.*
Oct. 6	2050	35	35	4.4	5.75	3.75	2.0	1.85	106	580
" 7	2034	35	70	3.8	5.2	3.68	1.52	2.4	109	670
" 8	2020	35	105	3.3	4.8	3.16	1.64	1.9	121	830
" 9		0								
" 10	1980	35	140	4.3	4.9	3.11	1.79	1.71	112	930
" 11	1970	30	170	3.1	4.27	2.57	1.70	1.5	115	1100
" 12	1980	25	195	2.9	3.78	2.23	1.55	1.44	136	1400
" 13	1940	38	233	2.0	3.5	2.45	1.05	2.3	154	1520
" 14	1940	35	268	1.5	3.32	2.25	1.07	2.1	192	1850
" 15	1920	35	303	1.18	2.95	1.69	1.26	1.34	224	2540
" 16	.	0								
" 17	1920	35	338	1.59	3.15	1.89	1.26	1.4	198	2340
" 18	1925	35	373	1.05	2.80	1.45	1.35	1.1	294	3400
" 19	1925	10	383	0.95	2.2	1.15	1.05	1.1	310	4120
" 20	1910	0	383	1.2				.		
" 21	1860	10	393	1.54	3.1	1.9	1.2	1.6	380	3750
" 22	1750	10	403	1.67	3.71	2.21	1.5	1.5	320	2800
" 23	1640	0	403	2.4						
" 24	1530	10	413	2.8	4.27	2.71	1.56	1.7	255	2300
" 25	1450	0	413	3.2						
" 26	1460	10	423	3.4	4.27	2.86	1.41	2.0	181	1800
" 27	1500	0	423	3.7				.		
" 28	1520	10	433	3.95	4.61	3.05	1.56	1.9	158	1250

Died Oct. 29.

It is seen from Table IV that there is a definite correlation to be observed between the fall of the serum proteins and the rise in the serum lipids. The concentration of the lipids in the blood does not have as intimate a relationship to the decrease in the number

of the red blood corpuscles as was previously supposed, but follows more closely the fall in the total serum protein.   Human pathology furnishes us with examples of this fact.   In spite of the enormous
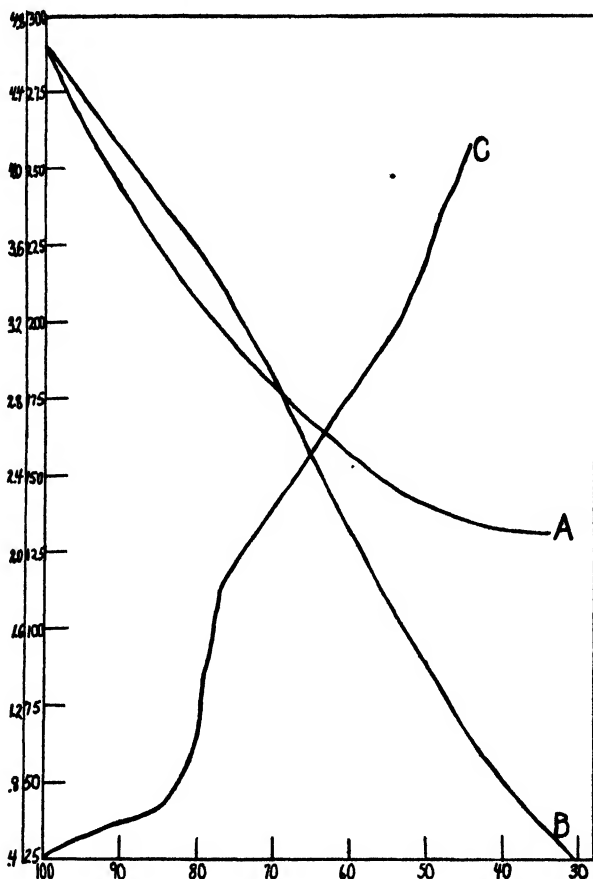
CHART I.   Relation of osmotic pressure to lipid concentration. *A*, in rabbit; *B*, dilution *in vitro*; *C*, lipid content.

decrease in the red blood cell count in pernicious anemia, there is no lipemia because the serum protein is not lessened.   It is in fact characteristic of this disease that the fat depots are well filled. Otherwise in the case of nephrosis and the nephrotic type of glo-

merulonephritis, even though there may be no anemia, yet following the loss of serum proteins in the urine, there is a great increase in the lipid content of the blood. This rise in total serum lipids after bleeding is analogous chemically to that of nephrosis, in that the total fatty substances are increased both relatively and absolutely much more than the cholesterol, the former rising

TABLE V.

Relation of Serum Lipids and Proteins to Osmotic Pressure.

Date.	Total red blood cells.		Total protein.	Albumin.	Globulin.	Albumin.globulin.	Cholesterol.	Total lipids.	Osmotic pressure.	Osmotic pressure per gm. protein.	Phosphorus.	Calcium.
1928	*cc.*	*mil-lion*	*gm. per cent*	*gm. per cent*	*gm. per cent*		*mg. per 100 cc.*	*mg. per 100 cc.*	*mm.*	*mm.*	*mg. per 100 cc.*	*mg. per 100 cc.*
Apr. 19	35	4.1	6.71	4.45	2.26	1.97	118	410	290	43.2	3.9	14.1
" 20	70											
" 21	100	3.5	6.51	4 32	2.19	1.97	122	412	268	41.2	4 0	14.2
" 23	130	4.1	6.84	4.64	2.20	2.1	120	392	273	40.0	4.0	14.1
" 24	160	3.3	6.11	3.96	2.15	1.85	112	515	242	39.7	3.8	14.0
" 25	190	2.6	6.06	3.95	2.11	1.87	131	621	237	39.1	3.8	14.0
" 26	220	2.1	6.21	4.05	2.16	1.87	119	724	242	39.0	3.7	14.0
27	240	2.0	6.14	4 06	2.08	1.95	128	601	233	38.0	3.8	14.1
" 28	275	1.7	5.67	3.67	2.00	1.84	145	622	212	37.4	4.3	13.7
" 30	310	1.9	5.51	3.57	1.94	1.84	171	1210	204	37.0	5.2	13.4
May 1	345	1.4	5.21	3.31	1.90	1.74	260	1872	200	38.4	5.0	13.2
" 2	385	1.25	4.47	2 75	1.72	1.60	287	2412	173	39.0	5.2	13.0
" 3	420	1.15	3.61	2.14	1.47	1.46	315	3215	147	40.8	5.0	13.0
" 4	455	1.0	3 23	1.95	1.28	1.52	345	3891	140	43.2	5.4	12.8
" 5	490	1.1	3.13	1.89	1.24	1.50	392	4041	141	45.1	5.8	12.7

to 10 times its initial value, the latter increase being only approximately 3- or 4-fold.

With the decrease in the concentration of the serum proteins, we see that the ratio of albumin to globulin is lowered, as is the case in nephrosis. Since at no time could any albumin be found in the urine, we must assume that the lowering of the albumin-globulin ratio is to be attributed to the more rapid regeneration of the globulin, as shown by Whipple (14) and his coworkers.

Thus we find that the three factors working toward a lowering of the osmotic pressure of the blood after hemorrhage or during the course of nephrosis are (a) the decrease in actual protein concentration, (b) the decrease in the osmotic pressure per gm. of protein with the increasing dilution of the serum, (c) the actual loss in number of molecules through a greater loss of the smaller albumin molecule through the kidney in nephrosis or because of the slower regeneration of the albumin molecule after bleeding.

3. Relation of Lipids, Proteins, and Osmotic Pressure of Blood.

The intimate relationship between the fall of the proteins, with its consequent lowering of the osmotic pressure of the blood and the rise of the serum lipids, suggests that these lipids may be mobilized in an effort to work against the falling osmotic pressure. If there were such a compensatory lipemia we would expect that the serum containing a relatively high proportion of these fatty substances should show a higher osmotic pressure than a similar serum at the same protein concentration made by dilution of the normal rabbit blood. This we measured day by day and the results have been plotted in a graph (Chart I). It can be seen that the curve of the rabbit serum shows a higher colloid osmotic pressure per gm. of protein than did the serum diluted *in vitro*. The two curves start to diverge as soon as the concentration of the lipoid substances in the blood reaches above 0.9 per cent which may be regarded as a high normal. It is to be noted that at this stage of our investigations we were unable to take into account the correction for the shift in the albumin-globulin ratio, but this correction in the curve of dilution *in vitro* would merely have made the difference in the osmotic pressure of the lipemic serum and the diluted normal serum more striking.

It is interesting to note (Table V) that there was an increase in the serum phosphorus and a decrease in the serum calcium as the protein content fell.

CONCLUSIONS.

1. The relationship between the concentration of proteins and the osmotic pressure of blood sera is expressed by the equation $p = e^{av}$ where p is the reciprocal of the osmotic pressure, v the

reciprocal of the protein concentration, and a a constant dependent on the particular serum; that is, that when the proteins decrease in arithmetical progression, the osmotic pressure decreases in geometric progression.

2. The lipemia that follows intensive hemorrhage in rabbits may perhaps be correlated with an effort of the organism to compensate for the decrease in osmotic pressure following the loss of serum proteins.

3. The osmotic pressure per gm. of protein is higher in lipemic blood than in blood diluted to the same concentration of protein, and this difference becomes apparent at the same time that the total lipid content of the blood starts to rise.

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ON INOSINIC ACID.

IV. THE STRUCTURE OF THE RIBOPHOSPHORIC ACID.

BY P. A. LEVENE AND T. MORI.*

(*From the Laboratories of The Rockefeller Institute for Medical Research,
New York.*)

There occur in nature ribonucleotides of two types. One is represented by inosinic acid, a ribonucleotide of animal origin, and the other is represented by adenylic and guanylic acids, which are components of yeast nucleic acid and thus are of plant origin. The principal distinction between the two types of nucleotides lies in the rate of hydrolysis of the phosphoric acid from the nucleosides. Yamagawa,[1] working in this laboratory, found the ratio between the constants of hydrolysis K (inos.):K (aden.) $= 1:3.5$. It was assumed that these differences are due to the differences in allocation of the phosphoric acid radicle. Levene and Jacobs[2] had previously concluded that in inosinic acid the phosphoric acid radicle is attached to carbon atom (5). This conclusion was reached on the basis of the result of oxidation of the phosphoribose obtained from inosinic acid. Under these conditions phosphoribonic and not phosphotrihydroxyglutaric acid was obtained. Recently Professor Robinson[3] has expressed the idea that ribose does not exist preformed in the nucleotides, but that it is formed during the process of hydrolysis through a Walden inversion. The assumption of Robinson presupposes the allocation of the phosphoric acid to one of the asymmetric carbon atoms, hence not to carbon atom (5). In view of this suggestion it seemed desirable to test the conclusion of Levene and Jacobs by

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* Fellow of the International Education Board.

[1] Yamagawa, M., *J. Biol. Chem.*, **43**, 339 (1920).

[2] Levene, P. A., and Jacobs, W. A., *Ber. chem. Ges.*, **44**, 746 (1911).

[3] Robinson, R., *Nature*, **120**, 44, 656 (1927). Levene, P. A., *Nature*, **120**, 621 (1927).

still another method.   Levene and Simms[4] have shown that from the curve of lactone formation of a sugar acid it is possible to differentiate between $<1,4>$ and $<1,5>$ lactones.   In a publication of Levene and Wolfrom[5] a plan was developed by which the allocation of a substituting group can be determined on the basis of the curve of lactone formation of a substituted sugar acid.

In the case of phosphoribonic acid the possibilities are the following.

I.   II.   III.
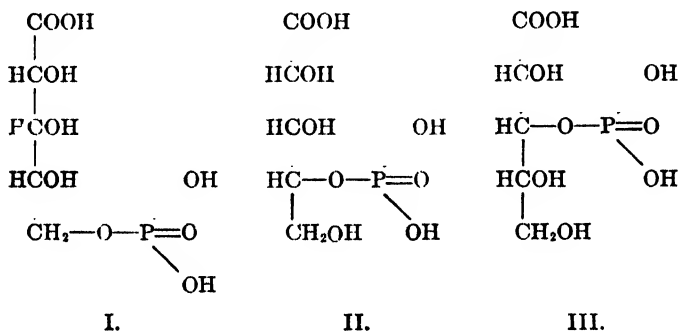
(1) The phosphoric acid radicle is attached to carbon atom (5) as in formula (I).   In this case a $<1,4>$ lactone will be formed; the course of its formation will be slow and the equilibrium will be established only after several days.

(2) The acid has the structure indicated in formula (II).   A $<1,5>$ lactone will be formed; the curve of the progress of lactone formation will show a rapid rise and equilibrium will be reached in less than 2 hours.

(3) The phosphoric acid radicle may be attached in position (3) as given in formula (III) or in position (2).   In this case both the $<1,4>$ and the $<1,5>$ lactones can form; therefore, the curve of lactone formation will have the character of that of mannonic lactone formation.

The phosphoribonic acid used in the present experiments was prepared by two methods, one sample by oxidation with nitric acid and the second by oxidation with barium hypoiodite, according to the method of Willstätter and Schudel as modified by

[4] Levene, P. A., and Simms, H. S., *J. Biol. Chem.*, **65**, 31 (1925).

[5] Levene, P. A., and Wolfrom, M. L., *J. Biol. Chem.*, **77**, 671 (1928).

Goebel.[6] As in the earlier experiments of Levene and Jacobs, the acid was obtained in the form of the neutral calcium salt having the composition $(C_5H_8O_9P)_2Ca_3$. In order to avoid a high hydrogen ion concentration of the solution, the monobasic salt of the composition $(C_5H_{10}O_9P)_2Ca$ was prepared. A solution

Changes in Rotation of 0.0825 N Monocalcium Phosphoribonate Solution.
$l = 2$ dm. $t = 25°$

Time.	α_D^{25}	$[\alpha]_D^{25}$
min.	*degrees*	*degrees*
15	−1.11	−12.68
30	−1.10	−12.58
45	−1.10	−12.58
hrs.		
1	−1.10	−12.58
2	−1.07	−12.23
3	−1.04	−11.89
4	−1.02	−11.66
6	−0.99	−11.32
8	−0.95	−10.86
10	−0.92	−10.52
24	−0.77	−8.80
32	−0.67	−7.66
48	−0.49	−5.60
56	−0.44	−5.03
72	−0.34	−3.89
81	−0.30	−3.43
98	−0.21	−2.40
103	−0.19	−2.17
120	−0.13	−1.49
129	−0.11	−1.26
146	−0.08	−0.91
168	−0.07	−0.80
192	−0.07	−0.80

of this salt was obtained by adding 2 equivalents of hydrochloric acid to the solution of the neutral salt.

The progress of lactone formation is given in Table I and from this table it is seen that the lactone formation proceeds very gradually, reaching an equilibrium in 150 hours. From the char-

[6] Goebel, W. F., *J. Biol. Chem.*, **72**, 801 (1927).

acter of the curve it follows, then, that the phosphoribonic acid obtained from inosinic acid has the phosphoric acid in position (5), as given in formula (I).

<div align="center">EXPERIMENTAL.</div>

Barium inosinate was prepared from fish meat extract[7] in the usual way.   This was hydrolyzed by boiling with 1 per cent hydrochloric acid, and the barium ribophosphate was isolated according to the directions of Levene and Jacobs.[2]
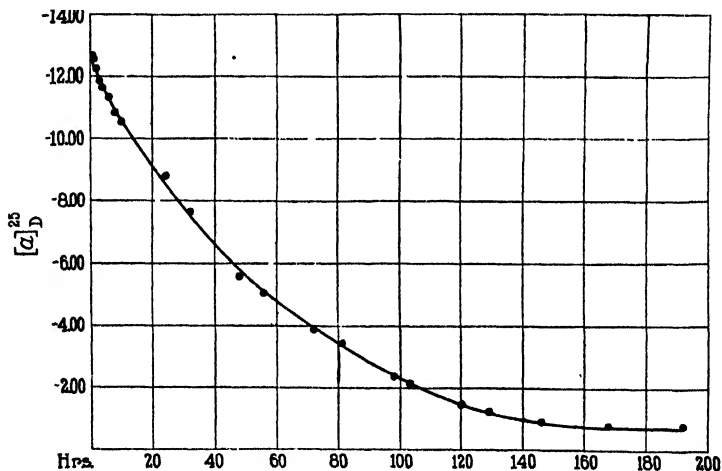
FIG. 1. Mutarotation of ribophosphoric acid.

The barium ribophosphate was then oxidized to phosphoribonic acid in two different ways.

First, the barium salt was treated with barium hypoiodite by the general method of Willstätter and Schudel as modified by Goebel.[6]

Second, the free ribophosphoric acid prepared from the barium salt was oxidized with nitric acid by exactly the same procedure as that described by Levene and Jacobs.[2]

In both cases the phosphoribonic acid thus obtained was isolated as its calcium salt.   The products were shown by analysis and

[7] We are indebted to Professor Yamagawa, Tokyo Imperial University, for the fish meat extract used in this work.

by determination of their rotations to be identical. The substance analyzed as follows.

5.265 mg. substance: 35.450 mg. ammonium phosphomolybdate.
0.1000 gm.   "   : 0.0272 gm. CaO.
$(C_5H_8O_9P)_2Ca_3$. Calculated. P 10.23, Ca 19.81.
                 Found.   " 9.77, " 19.44.

For the mutarotation experiment, 0.2500 gm. of the dried substance was treated with 2 equivalents of hydrochloric acid and the volume was made up to 5 cc. The optical rotation was observed in a 2 dm. tube at 25°. The results are shown in Table I and Fig. 1.

THE RELATION OF CHEMICAL STRUCTURE TO THE RATE OF HYDROLYSIS OF PEPTIDES.

IV. ENZYME HYDROLYSIS OF DIPEPTIDES.

BY P. A. LEVENE, LAWRENCE W. BASS, AND ROBERT E. STEIGER.

(*From the Laboratories of The Rockefeller Institute for Medical Research, New York.*)

In previous publications on this subject by Levene, Simms, and Pfaltz[1] and by Levene and Simms[2] the conclusion was reached that the rate of hydrolysis of di- and tripeptides is a function of the intrinsic dissociation constants of the two groups involved in the peptide linkage. A comparison was made between peptides formed from amino acids with free amino groups and peptides formed from amino acids methylated on the nitrogen atom. These series were selected for the reason that the dissociation constants of the two types of substances differ markedly. The progress of hydrolysis in the methylated peptides was complicated by the great tendency towards ketopiperazine formation. If this complicating circumstance had not existed, that is, if the rate of hydrolysis of unsubstituted peptides had been measured, the reaction should have been found to follow the course of an uncomplicated monomolecular reaction. To test the correctness of this conclusion, several peptides of this series have been prepared and have been subjected to the action of erepsin. The erepsin solution used in the older experiments was prepared from intestinal juice;[3] in the present experiments a more active enzyme solution was prepared according to directions of Northrop.

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[1] Levene, P. A., Simms, H. S., and Pfaltz, M. H., *J. Biol. Chem.*, **61**, 445 (1924); **70**, 253 (1926).

[2] Levene, P. A., and Simms, H. S., *J. Biol. Chem.*, **62**, 711 (1924–25).

[3] The intestinal juice was obtained from the Battle Creek Sanitarium through the kindness of Dr. Kellogg and Professor Boldyreff.

The results of our observations are summarized in Table I. From these results the following conclusions are warranted.

1. The reactions, under the conditions employed by us, follow the course of a monomolecular reaction.

2. In concentrations of substrate varying from 0.025 M to 0.500 M the rate of hydrolysis is nearly directly proportional to the concentration of enzyme. When a peptide composed of glycine and a d,l-acid is compared with one composed of glycine and a naturally occurring amino acid in regard to their rates of hydrolysis, the concentrations of the two substances should be in the ratio of 2:1; when one peptide is composed of two d, l-acids and the other of two optically active naturally occurring acids, the concentrations should be in the ratio of 4:1.

3. The rates of hydrolysis at the same concentration of glycyl-glycine, glycyl-dextro-valine, and glycyl-levo-leucine are of the same order of magnitude. This behavior was expected on the basis of previous experiments.

4. The behavior of peptides containing dextro-alanine differs from that of the peptides enumerated in (3). The highest rate of hydrolysis is observed in the case of dextro-alanyl-dextro-alanine; next in order is d,l-alanyl-glycine; the lowest rate is found for glycyl-d,l-alanine. A definite explanation of the exceptional behavior of alanine-containing peptides cannot be given at this stage of the work.

5. The presence of free glycine retards the hydrolysis of glycyl-glycine.

6. Peptides containing one amino acid enantiomorphous to that occurring naturally are not hydrolyzed by erepsin. This observation is in harmony with those of Abderhalden. On digestion of d,l-alanyl-glycine and similarly constituted peptides, the form containing the amino acid enantiomorphous to that occurring naturally remains intact.[4]

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[4] It should be noted in the case of d, l-alanyl-glycine (Table V, Column 3) that after the theoretical value of 3.00 cc. (representing complete hydrolysis of dextro-alanyl-glycine) has been reached, there is a very slow increase in the titer of the solution. This phenomenon may be explained by the assumption that the levo-alanyl-glycine, which remains unattacked by erepsin, is slowly racemized, either by the weak alkali or by an enzyme; the dextro-alanyl-glycine resulting from this partial racemization is then hydrolyzed by erepsin.

7. Glycyl-dextro-isovaline is not hydrolyzed by erepsin, although dextro-isovaline is configurationally related to the amino acids occurring in proteins.[5]

<div align="center">TABLE I.</div>
<div align="center">*Hydrolysis Constants of Peptides.*</div>

	Concentration.	$k \cdot 10^6$
	M	
Glycyl-glycine............. ....:..	0.025	74
"	0.050	47
"	0.100	26
"	0.500	4
" + 2 equivalents glycine..........	0.050	22
Glycyl-*d,l*-alanine.............................	0.100	87
"	0.200	40
Glycyl-dextro-valine.....	0.100	22
Glycyl-levo-valine.[6]...........................	0.100	0
Glycyl-dextro-isovaline.........................	0.100	0
Glycyl-levo-leucine.............................	0.100	33
d,l-Alanyl-glycine.[4]...........................	0.100	150
"	0.200	60
Levo-alanyl-glycine.[6]...........................	0.100	0
d,l-Alanyl-*d,l*-alanine..........................	0.100	8
Dextro-alanyl-dextro-alanine....................	0.100	150
Levo-alanyl-dextro-alanine.[6]....................	0.100	0

<div align="center">EXPERIMENTAL.</div>

<div align="center">*Preparation of Peptides.*</div>

The analyses of the peptides used in these experiments are summarized in Table II.

Glycyl-glycine,[7] glycyl-*d,l*-alanine,[8] glycyl-dextro-valine,[9] glycyl-

[5] Ehrlich, F., *Biochem. Z.*, **8**, 455 (1908).

[6] The very slow increase in the titer of these solutions is probably due to the presence of small quantities of the enantiomórphous peptides of the naturally occurring amino acids. Partial racemization may also occur (see foot-note 4).

[7] Fischer, E., and Fourneau, E., *Ber. chem. Ges.*, **34**, 2870 (1901).

[8] Fischer, E., *Ber. chem. Ges.*, **37**, 2489 (1904).

[9] Fischer, E., and Scheibler, H., *Ann. Chem.*, **363**, 138, 140 (1908).

dextro-isovaline,[10] glycyl-levo-leucine,[11] *d,l*-alanyl-glycine,[12] and levo-alanyl-glycine[13] were prepared by methods described in the literature.

d,l-Alanyl-d,l-alanine[14] was prepared from *d,l*-bromopropionyl chloride and *d,l*-alanine by Fischer's general procedure.

Dextro-alanyl-dextro-alanine[15] was obtained by fractional crystallization of crude *d,l*-alanyl-dextro-alanine.

Levo-alanyl-dextro-alanine[16] was obtained from crude *d,l*-alanyl-

TABLE II.

Analysis of Peptides.

	Sample No.	Calculated.			Found.			Moisture.
		C	H	N	C	H	N	
		per cent	per cent	per cent	per cent	per cent	per cent	per cent
Glycyl-glycine..............	2501	36.34	6.11	21.21	36.63	6.27	21.10	2.26
Glycyl-*d,l*-alanine.............	2516	41.07	6.90	19.18	41.11	6.58	19.31	1.48
d,l-Alanyl-glycine......	2502	41.07	6.90	19.18	40.95	6.56	19.29	2.10
Levo-alanyl-glycine.........	2503	41.07	6.90	19.18	40.38	6.75	19.36	2.90
Glycyl-dextro-valine..........	2526	48.24	8.10	16.09	48.22	8.22	16.10	1.60
Glycyl-levo-valine.............	2539	48.24	8.10	16.09	48.07	8.06	16.15	1.30
Glycyl-dextro-isovaline.......	2528	48.24	8.10	16.09	48.30	7.80	16.00	1.01
Glycyl-levo-leucine...........	2530	51.03	8.57	14.89	51.24	8.52	14.88	0.84
d,l-Alanyl-*d,l*-alanine.........	2504	44.97	7.55	17.50	44.88	7.32	17.27	0.70
Dextro-alanyl-dextro-alanine..	2505	44.97	7.55	17.50	45.12	7.35	17.42	1.59
Levo-alanyl-dextro-alanine...	2506	44.97	7.55	17.50	44.92	7.39	17.15	0

dextro-alanine by concentrating the mother liquors of crystallization of dextro-alanyl-dextro-alanine.

[10] Levene, P. A., and Steiger, R. E., *J. Biol. Chem.*, **76**, 305 (1928).

[11] Fischer, E., and Steingroever, J., *Ann. Chem.*, **365**, 167, 169 (1909).

[12] Fischer, E., *Ann. Chem.*, **340**, 128, 130 (1905).

[13] Fischer, E., *Ann. Chem.*, **340**, 166 (1905); also *Ber. chem. Ges.*, **40**, 507 (1907).

[14] For properties see Fischer, E., and Kautzsch, K., *Ber. chem. Ges.*, **38**, 2377 (1905).

[15] See Fischer, E., and Schulze, A., *Ber. chem. Ges.*, **40**, 952, 954 (1907). Fischer, E., *Ber. chem. Ges.*, **39**, 465 (1906).

[16] See Fischer, E., and Schulze, A., *Ber. chem. Ges.*, **40**, 952, 954 (1907). Fischer, E., and Raske, A., *Ber. chem. Ges.*, **39**, 3989 (1906).

Glycyl-levo-valine. Chloroacetyl-levo-valine. 23 gm. (0.2 mol) of levo-valine were dissolved in 100 cc. of 2.0 N sodium hydroxide (0.2 mol) and treated alternately under shaking with 45 gm. (0.4 mol) of chloroacetyl chloride (b.p. 106° at 762 mm.) and 240 cc. of 2.0 N sodium hydroxide (0.48 mol) under cooling in ice, each reagent being added in equivalent proportions. The time of introduction was about 1 hour. Addition of 56 cc. of 5.0 N hydrochloric acid (0.28 mol) yielded a precipitate of the chloroacetyl compound, which, after drying in a vacuum desiccator over phosphorus pentoxide, weighed 25 gm. (Yield, 65 per cent of the theory.) For analysis, the compound was recrystallized from 5 times its weight of boiling water and dried at 100° over sulfuric acid under diminished pressure. Melting point 112–113°.

0.1006 gm. substance: 0 1610 gm. CO_2 and 0.0574 gm. H_2O.
0.1000   5.20 cc. 0.1 N HCl (Kjeldahl).
0 1236   0.0926 gm. AgCl (Carius).
$C_7H_{12}O_3NCl$ (193.61). Calculated. C 43.41, H 6.25, N 7.24, Cl 18.32.
                           Found.   " 43 64, " 6.38, " 7.28, " 18.53.

$$[\ ]^{25}_{5461} = \frac{-1.26° \times 100}{4 \times 1.94} = -16.3° \text{ (in absolute alcohol)};$$

$$[\alpha]^{20}_{D} = \frac{-1.02° \times 100}{4 \times 1.94} = -13.1° \text{ (in absolute alcohol)};$$

0.4840 gm. in 25 cc. (0.1 M solution).

$$[\alpha]^{25}_{D} = \frac{-3 00° \times 100}{2 \times 10.00} = -15.0° \text{ (in absolute alcohol)};$$

1.0000 gm. in 10.0 cc. of solution.

Fischer and Scheibler[17] found for chloroacetyl-dextro-valine $[\alpha]^{20}_{D} = +15.8°$ ($\pm 0.2°$), employing a solution of 0.5051 gm. in absolute alcohol, the weight of the solution being 5.0115 gm.

Glycyl-levo-valine. 21 gm. of crude chloroacetyl-levo-valine were allowed to stand 3 days at room temperature with 210 gm. of ammonium hydroxide (sp. gr. 0.90). The solution was then concentrated to dryness under reduced pressure. The residue of crude dipeptide and ammonium chloride was dissolved in 35 cc. of hot water. 500 cc. of hot absolute alcohol were added. Crys-

[17] Fischer, E., and Scheibler, H., *Ann. Chem.*, **363**, 140 (1908).

tallization of the dipeptide, which set in immediately, was completed by cooling in ice. The crystals were washed with absolute alcohol. They weighed 15 gm. (Yield, 79 per cent of the theory.) For purification they were dissolved in 30 cc. of water in the presence of some norit. 300 cc. of absolute alcohol were added to the filtrate and the crystals were filtered off after standing 24 hours in ice. The product was washed with alcohol. The yield was 12.1 gm.

0.0877 gm. substance: 0.1546 gm. CO_2 and 0.0630 gm. H_2O.
0.0494 "   "   : 5.70 cc. 0.1 N HCl (Kjeldahl).
$C_7H_{14}O_3N_2$(174.17). Calculated. C 48.25, H 8.10, N 16.09.
                    Found.   " 48.07, " 8.06, " 16.15.

$$[\alpha]_D^{25} = \frac{+ 2.03° \times 100}{2 \times 5.00} = + 20.3° \text{ (in water)};$$

0.500 gm. in 10.0 cc. of solution.

Fischer and Scheibler[18] found for glycyl-dextro-valine $[\alpha]_D^{20} = -19.7°$ ($\pm 0.2°$), employing a solution of 0.4071 gm. in water, the weight of the solution being 4.1193 gm.

Preparation of Enzyme Solution.

Clean, fresh pig intestine (10 pounds), finely ground, was suspended in glycerol (3 liters). The suspension, which was kept at 12°, was stirred at frequent intervals to insure thorough extraction of the enzyme. After 48 hours the mixture was filtered through paper in a battery of funnels, and the filtrate was stored at 0°.

For the preparation of an aqueous solution, a suitable quantity of this glycerol solution was dialyzed in collodion bags against running water for 48 hours. The cloudy solution inside the bags was filtered, and the filtrate was adjusted to pH 8.0. The same solution was used throughout the experiments.

Procedure in Hydrolysis Experiments.

Samples of the peptides were weighed in volumetric flasks. To each sample were then added 0.3 equivalent of alkali and the volume of enzyme solution given in Tables III to V. The flasks were then filled to the mark with distilled water and placed

[18] Fischer, E., and Scheibler, H., *Ann. Chem.*, **363**, 141 (1908).

TABLE III.

Hydrolysis of Glycyl-Glycine by Erepsin.*

Experiment with: (1)	Control. (2)			0.025 M. (3)			0.050 M. (4)			0.100 M. (5)			0.500 M. (6)			0.050 M + glycine. (7)		
Glycyl-glycine, mols.	0.005			0.005			0.005			0.005			0.005			0.0025		
Glycine, mols.	0.005			0			0			0			0			0.005		
Alkali, equivalents.	0.3 (glycine).			0.3			0.3			0.3			0.3			0.3 (glycine + glycyl-glycine).		
Erepsin solution, cc.	5.0			20.0			10.0			5.0			1.0			5.0		
Total volume, cc.	50.0			200.0			100.0			50.0			10.0			50.0		
Samples, cc.	5.0			20.0			10.0			5.0			1.0			5.0		
	t	V	$k \cdot 10^3$	t	V	$k \cdot 10^3$	t	V	$k \cdot 10^3$	t	V	$k \cdot 10^3$	t	V	$k \cdot 10^3$	t	V	$k \cdot 10^3$
	hrs.	cc.		hrs.	cc.		hrs.	cc.		hrs.	cc.		hrs.	cc.		hrs.	cc.	
	0	1.75	0	0	1.97		0	1.84		0	1.78		0	1.83		0	2.60	
	1.0	1.75	0	1.0	2.35	72	1.0	2.08	44	1.0	1.92	25	1.0	1.87	(7)	1.0	2.67	25
	5.0	1.76	0	2.0	2.65	69	2.0	2.32	46	2.0	2.05	25	2.0	1.90	(6)	2.0	2.73	24
	23.0	1.74	0	4.0	3.21	74	4.0	2.72	47	4.0	2.31	26	4.0	1.93	4	3.0	2.77	21
	48.0	1.75	0	5.0	3.43	76	5.0	2.88	47	6.0	2.57	27	6.0	1.96	4	4.25	2.84	22
				6.0	3.60	76	6.0	3.05	48	8.0	2.76	27	24.0	2.35	4	6.0	2.90	20
				7.0	3.74	77	7.0	3.22	49	24.0	3.71	27	48.0	2.73	4			
				8.0	3.85	76	8.0	3.36	50	47.0	4.04	(23)						
				24.0	4.47		24.0	4.13		73.0	4.19							
										102.0	4.21							
Average $k \cdot 10^3$.			0			74			47			26			4			22

* In Tables III, IV, and V, values of $k \cdot 10^3$ in parentheses have not been included in calculating average values.

TABLE IV.

Hydrolysis of Glycyl-Peptides by Erepsin.

Experiment with: (1)	Glycyl-d,l-alanine, 0.200 M. (2)			Glycyl-d,l-alanine, 0.100 M. (3)			Glycyl-dextro-valine, 0.100 M. (4)			Glycyl-levo-valine, 0.100 M. (5)			Glycyl-dextro-isovaline, 0.100 M. (6)			Glycyl-levo-leucine, 0.100 M. (7)		
Peptide, *mols.*	0.005			0.005			0.005			0.005			0.005			0.005		
Alkali, *equivalents.*	0.3			0.3			0.3			0.3			0.3			0.3		
Erepsin solution, *cc.*	2.5			5.0			5.0			5.0			5.0			5.0		
Total volume, *cc.*	25.0			50.0			50.0			50.0			50.0			50.0		
Samples, *cc.*	2.5			5.0			5.0			5.0			5.0			5.0		
	t *hrs.*	*V* *cc.*	$k \cdot 10^3$	*t* *hrs.*	*V* *cc.*	$k \cdot 10^3$	*t* *hrs.*	*V* *cc.*	$k \cdot 10^3$	*t* *hrs.*	*V* *cc.*	$k \cdot 10^3$	*t* *hrs.*	*V* *cc.*	$k \cdot 10^3$	*t* *hrs.*	*V* *cc.*	$k \cdot 10^3$
	0	1.76		0	1.74		0	1.74		0	1.76		0	1.75		0	1.72	
	1.0	1.85	(33)	0.5	1.88	(104)	0.5	1.84	(35)	1.0	1.75	0	0.5	1.75	0	0.5	1.82	35
	2.0	1.98	42	1.0	1.98	(93)	1.0	1.89	(27)	5.5	1.79	0	1.0	1.74	0	1.0	1.89	31
	3.0	2.07	41	1.5	2.06	86	2.0	2.00	24	7.5	1.79	0	2.0	1.74	0	2.0	2.05	31
	4.0	2.13	38	2.0	2.15	87	3.0	2.08	21	24.0	1.81		3.0	1.76	0	3.0	2.21	32
	5.0	2.22	40	2.5	2.23	87	4.0	2.17	20	55.0	1.87		4.0	1.77	0	4.0	2.35	32
	6.0	2.28	39	3.25	2.35	89	5.5	2.36	22				5.5	1.77	0	5.5	2.58	33
	8.0	2.41	40	6.0	2.76	(123)	7.0	2.49	22				7.0	1.80		7.0	2.78	34
	23.0	2.97		25.0	3.24		26.0	3.70	(26)				26.0	1.89		26.0	3.94	36
Average $k \cdot 10^3$.	40			87			22			0			0			33		

TABLE V.

Hydrolysis of Alanyl-Peptides by Erepsin.*

Experiment with: (1)	d,l-Alanyl-glycine, 0.200 M. (2)			d,l-Alanyl-glycine, 0.005 M. (3)			Levo-alanyl-glycine, 0.100 M. (4)			d,l-Alanyl-d,l-alanine, 0.100 M. (5)			Dextro-alanyl-dextro-alanine, 0.100 M. (6)			Levo-alanyl-dextro-alanine, 0.100 M. (7)		
Peptide, *mols.*	0.005			0.005			0.005			0.005			0.005			0.005		
Alkali, *equivalents.*	0.3			0.3			0.3			0.3			0.3			0.3		
Erepsin solution, cc.	2.5			5.0			5.0			5.0			5.0			5.0		
Total volume, cc.	25.0			50.0			50.0			50.0			50.0			50.0		
Samples, cc.	2.5			5.0			5.0			5.0			5.0			5.0		
	t (hrs.)	V (cc.)	$k \cdot 10^5$	t (hrs.)	V (cc.)	$k \cdot 10^5$	t (hrs.)	V (cc.)	$k \cdot 10^5$	t (hrs.)	V (cc.)	$k \cdot 10^5$	t (hrs.)	V (cc.)	$k \cdot 10^5$	t (hrs.)	V (cc.)	$k \cdot 10^5$
	0	1.83		0	1.76		0	1.78		0	1.78		0	1.73		0	1.75	
	1.0	1.98	56	0.5	1.96	15_1	1.0	1.87	0	1.0	1.81	(13)	0.5	2.05	(11_9)	1.0	1.78	0
	2.0	2.14	62	1.0	2.11	14_8	2.0	1.94	0	2.0	1.82	(11)	1.0	2.42	14_0	2.0	1.79	0
	3.0	2.25	59	1.5	2.24	14_0	5.0	1.96	0	5.0	1.86	9	1.5	2.74	15_0	5.0	1.78	0
	4.0	2.36	60	2.25	2.41	16_1	25.0	2.02		6.0	1.86	6	2.0	3.03	16_0	25.0	1.92	
	5.0	2.42	65	3.0	2.63	16_0	53.0	2.10		24.0	1.97	6	2.5	3.27	16_4	53.0	2.07	
	6.0	2.53	59	4.0	2.89	(25_4)				25.0	2.04	8	3.25	3.64	(19_1)			
	7.0	2.62	62	6.0	2.92	(19_0)				47.0	2.18	8	6.0	4.09	(20_7)			
	23.0	3.02		25.0	3.02					53.0	2.24	9	25.0	4.16				
				47.0	3.23					73.0	2.41	(15)						
				73.0	3.47					102.0	2.57							
				102.0	3.55													
Average $k \cdot 10^5$.			60			15_1			0			8			15_4			0

* The inferior numbers are used to indicate figures which are not significant.

in a thermostat at 37°.   Samples, withdrawn at given intervals, were titrated (0.2 N NaOH) by Sørensen's formol titration method, with thymolphthalein as an indicator.

The reaction constants were calculated by means of the equation

$$kt = \log_{10} \frac{a}{a - x}$$

In the calculations a was taken as the theoretical quantity of peptide susceptible to hydrolysis.   The data are given in Tables III to V.

Control experiments with 0.100 M glycyl-glycine were run for each series of hydrolyses; the hydrolysis constants of these controls were identical within the limits of error.

Hydrolysis of d,l-Alanyl-Glycine.

A solution of 0.005 mol of peptide, 0.3 equivalent of alkali, and 5.00 cc. of enzyme solution was made up to a volume of 25.0 cc. A sample of 2.50 cc. required (formol titration) 1.76 cc. of 0.2 N alkali.   A sample of 5.00 cc. was neutralized with the theoretical quantity of hydrochloric acid, filtered, and diluted to 10.0 cc. The rotation in a 1 dm. tube was $\alpha_D^{25} = 0°$.

The original solution was then allowed to stand 20 hours at 37°.   A sample of 2.50 cc. required (formol titration) 2.98 cc. of 0.2 N alkali.   A sample of 5.00 cc. was neutralized with the theoretical quantity of hydrochloric acid, filtered, and diluted to 10.0 cc.   The rotation in a 1 dm. tube was $\alpha_D^{25} = -0.38°$.

The calculated rotation of a mixture of levo-alanyl-glycine and dextro-alanine equivalent to the above solution is $\alpha_D^{25} = -0.36°$.

SUPPLEMENTARY NOTE ON THE NEW FERRICYANIDE METHOD FOR BLOOD SUGAR.

By OTTO FOLIN.

(From the Biochemical Laboratory of Harvard Medical School, Boston.)

Anna L. Post, of the Metropolitan Life Insurance Company, has called to my attention the fact that the colorimetric ferricyanide method[1] for blood sugar gives the low sugar values, which I had reported, only when the dilute tungstic acid solution employed for the protein precipitation is fresh.

I verified her observation to a certain extent in July, but could obtain no deterioration of the tungstic acid when the investigation was resumed in the autumn. As a result of further cooperative studies by Miss Post and myself, it finally became clear that the determining factors were sunlight and toluene. Miss Post, following the original directions, always added toluene, and the Metropolitan laboratory is exposed to much direct sunlight (through the roof), while I did not use toluene, and I kept the solutions in a comparatively dark part of the laboratory. By moving my solutions to a window with southeast exposure and adding toluene, I found that my solutions also began to deteriorate. In a week, they began to give blood sugar values 15 to 18 per cent higher than those obtained by the use of freshly prepared tungstic acid solutions. On the other hand, tungstic acid solutions which had been kept in a dark cupboard without toluene gave perfectly water-clear extracts and correct blood sugar values for a period of at least 5 months.

The deteriorated tungstic acid solutions curiously enough seem to precipitate the blood proteins in rather more compact form, thus giving the impression that perhaps the precipitation is less complete. This is not the case, however, for no further precipi-

[1] Folin, O., *J. Biol. Chem.*, **77**, 421 (1928).

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tation could be secured from the extracts.   The solutions preserved with toluene and exposed to light invariably develop reducing properties and give gradually increasing color in blank experiments.   They also increase the intensity of color obtained from glucose to a much   greater extent than is indicated by their own reducing power.   In other words, toluene in the presence of tungstic acid is decomposed by light, partly into reducing products, and partly into products which are oxidized in the presence of some other easily oxidizable substance (glucose).   In the absence of tungstic acid, toluene does not seem to be similarly decomposed by light.   The investigation on the combined effects of light and tungstic acid is being continued.

It is not to be supposed that the destructive effect of light in the presence of tungstic acid is specific for toluene.   Probably all organic matter which might be present as impurity in the tungstic acid would be similarly affected; hence, even when no toluene is used, the tungstic acid solutions should not be exposed to too much light.

A number of other private communications have been received from persons who have had trouble with the new sugar method.   Some have obtained extremely low values.   And some have thought that there must be a flaw in the method, because they cannot secure dependable sugar values from the sugar standard when the concentrated sugar standard is diluted with tungstic acid solution instead of with water.

The latter point represents a demand that the method shall work in the presence of more acid than should ever be present in blood filtrates.   The blood proteins precipitate the tungstic acid almost as completely as the tungstic acid precipitates the proteins and, consequently, the extracts are almost neutral.   The observation is not without value, however, for it serves to emphasize the fact that the alkali used in this method has very little buffer capacity.   No attempt has been made to get an alkali with much buffer value, because there should be very little need for such buffer effect in this method.

Those who have obtained little or no blood sugar by the micro method have almost certainly been using grossly erroneous sulfuric acid for the preparation of the dilute tungstic acid.   When the 2/3 N sulfuric acid is much too strong, one result will be blood

filtrates which contain so much sulfuric acid that the carbonate-cyanide mixture may not give the requisite alkalinity for the reduction. On the other hand, if the sulfuric acid is much too weak, the result will be incomplete precipitation of the proteins, although the result is probably always accompanied by turbid extracts and, therefore, easily detected. There is ample, but not unlimited, room for analytical errors in the preparation of the 2/3 N sulfuric acid.

In the original paper very little was said about the keeping quality of the different reagents used in the new blood sugar method, and this note is intended to give such information as has since been obtained on these points.

1. Dilute Tungstic Acid Solution.—In response to personal inquiries many have been advised to use only freshly prepared tungstic acid solutions. This is not necessary, provided that no preservative is used and that the reagent is not exposed to too much light. Uncontaminated tungstic acid solutions may be used just as long as they continue to give perfectly water-clear blood extracts. It is probably best occasionally to test the reagent for reducing materials by making a blank test on 2 cc. of the reagent plus 2 cc. of water. If any blue color at all is obtained the reagent should be discarded.

2. Potassium Ferricyanide Solution.—Contrary to my expectation, this solution if kept completely protected from light (in a brown bottle kept in a dark closet) seems to keep for a very long time. My present stock solution is now 4 months old and it is still perfectly good. The reagent in daily use is also kept in a brown bottle, and is stocked only with about enough for 1 week's use.

3. Sodium Cyanide-Carbonate Solution.—This reagent, prepared as described in the original paper, also keeps surprisingly well. My 4 months old solution is still as satisfactory as freshly prepared solutions. In this case I rather expected the cyanide to diminish gradually and finally disappear. The importance of the cyanide lies in its power to increase greatly the color obtained from a given minute quantity of sugar.

4. Ferric Iron Solution.—From the beginning, this reagent has given me a great deal of trouble. The samples of gum arabic available in different laboratories have clearly been quite different. Some workers have reported that even with freshly prepared rea-

gents, the Prussian blue did not remain dispersed long enough to permit the making of the color comparison, while others have obtained such unfortunate results with solutions less then 2 weeks old.   A part of these difficulties may be due to too long heating, for heat seems to diminish greatly the keeping quality of gum arabic solutions.   My own reagents have always kept for several weeks, and I know that one solution, in Dr. Joslin's laboratory, kept for over 4 months.   Even when the gum arabic is kept separately in pure water solution, some workers have found that the protective colloidal properties disappear in a few weeks or less.   My own solutions, made 4 months ago, are still perfectly good.   When kept in the cold room ($3-4°$) our regular acid solutions have shown no signs of deterioration at the end of 2 months.

With Dr. J. A. Cavalcanti, I have looked into the problem of finding some better protective colloid than gum arabic, and we believe that in gum ghatti we have found one which does not hydrolyze nearly so easily as gum arabic and which, moreover, is several times more effective than gum arabic as a protective colloid.   Our gum is soluble gum ghatti No. 2, as sold by Howe and French, in Boston.[2]

Gum ghatti is a crude looking product and it is undoubtedly a natural mixture of two or more ingredients, one of which seems to be almost insoluble in cold water and extremely slowly soluble in hot water.   It is a very interesting material, but at the present time we are concerned only with its practical use as a protective colloid for Prussian blue.

Gum ghatti comes from the stems of *Anogeissus lætifolia*, a tree indigenous to India and Ceylon. According to the Dispensatory of the United States (Philadelphia, 21st edition, 1926, pp. 543–544), the gum is entirely soluble in water, and it is referred to by dealers as soluble gum ghatti. None of the samples tested here has been entirely soluble in water.

The preparation of gum ghatti solutions suitable for incorporation in the acid iron phosphate reagent has not been without its difficulties.   It is not practical to try to dissolve the gum directly in a flask of boiling water, because the gum will stick to the bottom of the flask and get burned.   If heated in water on a steam bath

[2] Gum ghatti can also be obtained from Eimer and Amend, New York.

the gum also sticks to the bottom, and because of the insoluble fraction which swells up like agar, it is rather uncertain how long one should heat it; if heated too long there might occur, as with gum arabic, considerable destruction of the protective colloidal properties. Experienced chemists can, of course, easily devise means of making the solution either by boiling or by help of the steam bath, but we wanted a method suitable for less experienced workers. The following process should meet the needs of all.

Fill a liter cylinder to the mark with cold water. Push into this cylinder a circular piece of window screening (galvanized iron or copper) large enough to form a bowl well below the surface of the water. Transfer 20 gm. of the gum to the wire screen bowl, cover the cylinder, and set aside for 18 to 24 hours. Larger cylinders may, of course, be used if much solution is wanted; I usually take a 4 liter cylinder and 80 gm. of the gum. Solution begins immediately, as can be seen by inspection, and nearly all that will dissolve in cold water is obtained at the end of about 24 hours. Most of the impurities and the *insoluble* gum remain on the screen and are thus easily removed. At the end of the specified time, filter, or strain through a piece of clean laboratory toweling, to remove the dirt which has passed through the screen.

Dissolve 5 gm. of ferric sulfate, ordinary ferric sulfate with 7 molecules of water of crystallization, together with 75 cc. of 85 per cent phosphoric acid in 100 cc. of water, by the aid of heat. Cool and mix with the gum ghatti solution. The turbidity which occurs at this stage is not important.

The reagent containing iron, phosphoric acid, and a protective colloid thus obtained is not entirely satisfactory without some further treatment. The gum ghatti may be a mixture of several ingredients, and the solutions made from it contain a certain amount of material capable of reducing ferricyanide in an acid medium. To demonstrate this point one needs only to add 3 cc. of the reagent to a test-tube containing 4 cc. of water and 1 cc. of the potassium ferricyanide solution. Within the required waiting period, 5 minutes, more or less Prussian blue is produced, a great deal more than is obtained from a corresponding iron phosphate solution which contains no gum or from a solution containing gum arabic.

The unknown reducing materials in the reagent are fortunately very easily destroyed by the addition of a little potassium permanganate. Add 1 per cent permanganate solution, at first 5 cc., and later about 3 cc., at a time, and continue until the pink color of permanganate remains perceptible for at least 5 to 10 minutes. My gum ghatti requires about 0.4 cc. of permanganate per gm. or about 30 cc. for 4 liters of reagent, and the reagents thus oxidized give scarcely a trace of blue color in the course of 15 minutes when 3 cc. are added to water containing 1 cc. of pure potassium ferricyanide solution and 1 cc. of carbonate cyanide solution.

The oxidation with permanganate must not be omitted, because in dealing with natural products like gums one can never be sure that some batches may not contain more disturbing impurities than others, to say nothing of the possible traces of ferrous salt in the ferric sulfate. In fact, I now use and recommend exactly the same process as is described here, including the oxidation with permanganate, for the preparation of the ferric iron reagent even when gum arabic is used as the protective colloid.

I should have liked to delay the publication of this note for more prolonged observations on the keeping quality of the gum ghatti solutions, but in view of the difficulties encountered by others with gum arabic and tungstic acid solutions, it seems better to publish it now. Our present experience with gum ghatti may be summarized in the statement that as a protective colloid it is at least 5 times as effective as gum arabic and the acid iron phosphate solutions containing gum ghatti have shown no signs of deterioration at the end of 2 months. The Prussian blue by which one measures the sugar reduction will remain in a clear uniform dispersion practically indefinitely (weeks), but the color comparison must be finished promptly as directed in the original paper.

THE DISSOCIATION CONSTANTS OF CERTAIN AMINO ACIDS.*

By PAUL L. KIRK and CARL L. A. SCHMIDT.

(From the Division of Biochemistry, University of California Medical School, Berkeley.)

Various attempts (1-4) have been made to correlate the acid-
and base-combining capacity of proteins with the content of
certain of the amino acids. As better analytical data have become
available, this correlation has become closer, but it is made some-
what uncertain by lack of accurate knowledge of the ability of
certain amino acids to combine with acid and with base. Ulti-
mately, this correlation must also take into consideration the
stereochemical structure of the protein molecule. It has been
previously pointed out (5) that values for the dissociation constants
of oxyproline, β-hydroxyglutamic acid, and serine are still lacking.
To this list we can add isoleucine and norleucine. To make the
list complete there should also be added several other recently
discovered amino acids. Our knowledge of these is, at the present
time, too fragmentary to warrant attempting work of the nature
herein described. Moreover, the dissociation constants for glu-
tamic acid were determined by somewhat inadequate methods (6).
Harris (7) has published the dissociation constants for valine,
but without titration curves, and with an omission in the tables,
making it desirable to repeat the determination of the constants
for this amino acid.[1]

[1] During the preparation of this paper, Simms (8) has published the
constants for valine and glutamic acid. We are, however, unable to find
the detailed data in the literature.

We accordingly have attempted to fill these gaps in our knowledge of the acid and base strength of the amino acids. It might be pointed out that such knowledge is useful, not only in elucidating our concepts of the behavior of the protein molecule, but also in physicochemical studies in which the properties of the amino acid are of direct concern, as for example, the work of Foster and Schmidt (9).

EXPERIMENTAL.

The glutamic acid used in this investigation was prepared from "Ajinomoto" by the method of Schmidt and Foster (10). Racemic valine, racemic norleucine, and d-isoleucine were Eastman Kodak products. All were recrystallized before use. A second sample of carefully purified valine, which had been isolated by Dr. T. B. Osborne, was kindly supplied to us by the Connecticut Agricultural Experimental Station. A second sample of isoleucine was obtained from the Special Chemicals Company. It was recrystallized before use. Oxyproline was supplied to us by Dr. J. Kapfhammer. It was prepared according to the method described by Kapfhammer and Eck (11). The analysis showed it to be a very pure product. Serine was synthesized by the method of Leuchs and Geiger (12) and was recrystallized a number of times. The β-hydroxyglutamic acid was prepared from casein by a combination of the transport method of Foster and Schmidt (9) and the method described by Dakin (13). It was further purified by the method suggested by Dakin. The preparation contained a small amount of ash and a correction for this was applied to the titration curve.

All samples were dried over phosphorus pentoxide before weighing. Amino nitrogen estimations on all samples which were prepared or purified by us gave, within experimental limits, theoretical values.

The titration curves were determined by use of the hydrogen electrode. The electrode vessel was of the Clark type. Hydrogen was generated electrolytically from sodium hydroxide solution between nickel electrodes, passed over concentrated sulfuric acid to remove sodium hydroxide spray, then passed over hot platinized asbestos to remove oxygen, bubbled through water, and led into the electrode vessel. The comparison half-cell was a 1.0

ɴ KCl calomel cell frequently checked against other similar cells of various ages. This was connected to the hydrogen electrode by means of saturated potassium chloride. All cells were kept at 25° in an air thermostat. The potentials were measured by means of a Leeds and Northrup type K potentiometer,[2] a sensitive light beam galvanometer being used for zero point instrument.
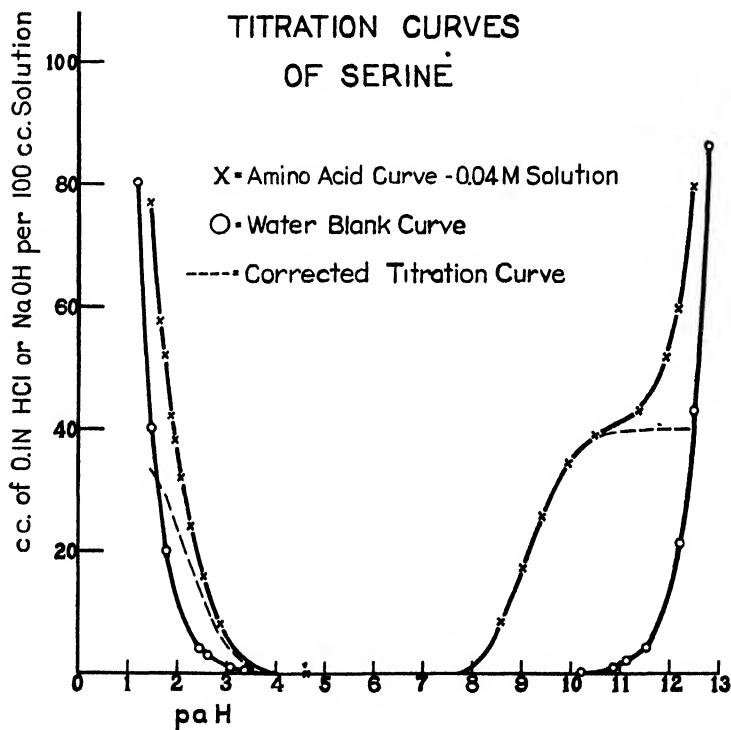

FIG. 1.

Potentials were reduced to pa_H by means of the Schmidt and Hoagland tables (14) which use the values of 0.336 volt for the decinormal KCl calomel electrode, and 0.283 volt for the normal KCl calomel electrode. It might be pointed out that these values

are in much better agreement with recent work (15–17) than the older Sörensen values (18).   The pa_H in all solutions which were acid in reaction was estimated by means of the quinhydrone electrode, with the 1.0 N KCl calomel electrode as the reference
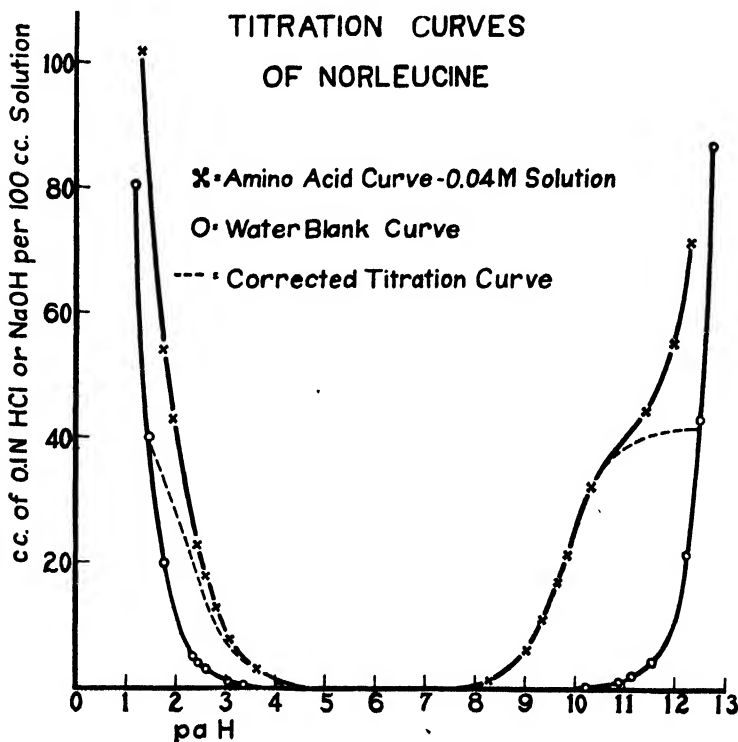
FIG. 2.

cell, as well as by means of the hydrogen electrode.   The agreement between the two methods was in all cases very satisfactory.

RESULTS.

The curves of Figs. 1 to 7 show the results of titration of glutamic acid, serine, isoleucine, norleucine, valine, and β-hydroxyglutamic acid with acid and with base.   The dotted curves show

TITRATION CURVES
OF ISOLEUCINE

X = Amino Acid Curves
① Special Chemical Co. Preparation - 0.04 M Solution
② Eastman Kodak Co. Preparation - 0.031 M Solution

O = Water Blank Curve

---- Corrected Titration Curve
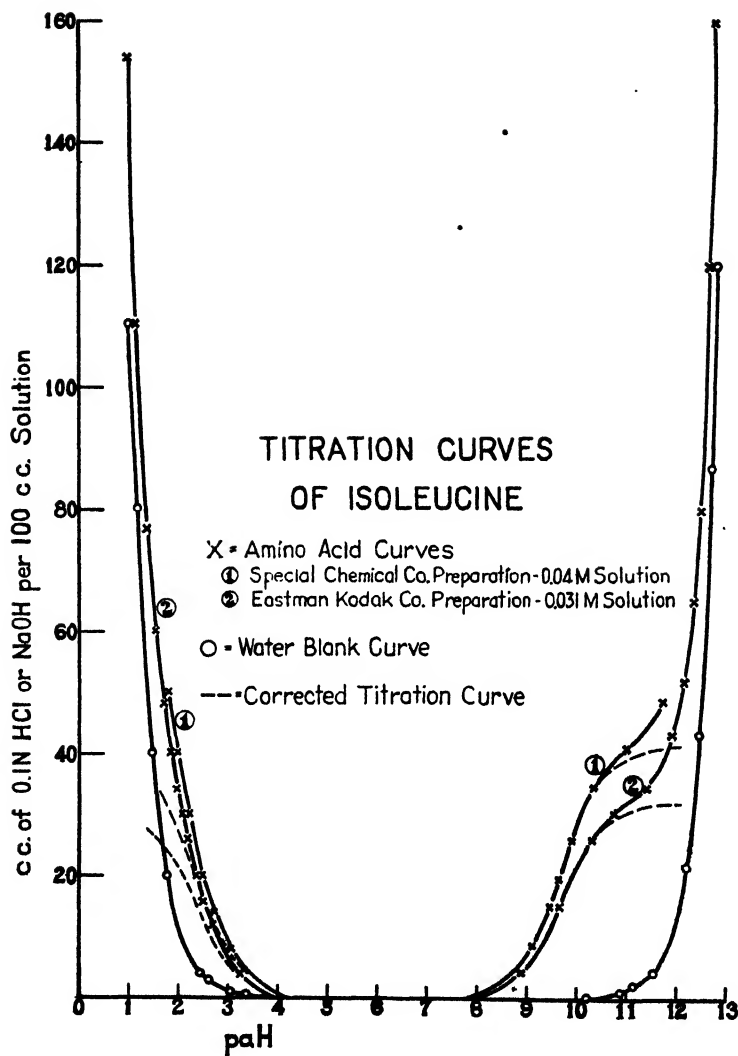
FIG. 3.

the correction for the water blank. In accord with the simple Henderson-Hasselbalch equation

$$pa_H = pK'_a + \log \frac{salt}{acid}$$

the reading of pa_H of the curve at the point of half neutralization with base would represent the value of pK'_a where pK'_a repre-

TITRATION CURVES
OF VALINE

X = Amino Acid Curve - 0.04 M Solution
O = Water Blank Curve
---- = Corrected Titration Curve
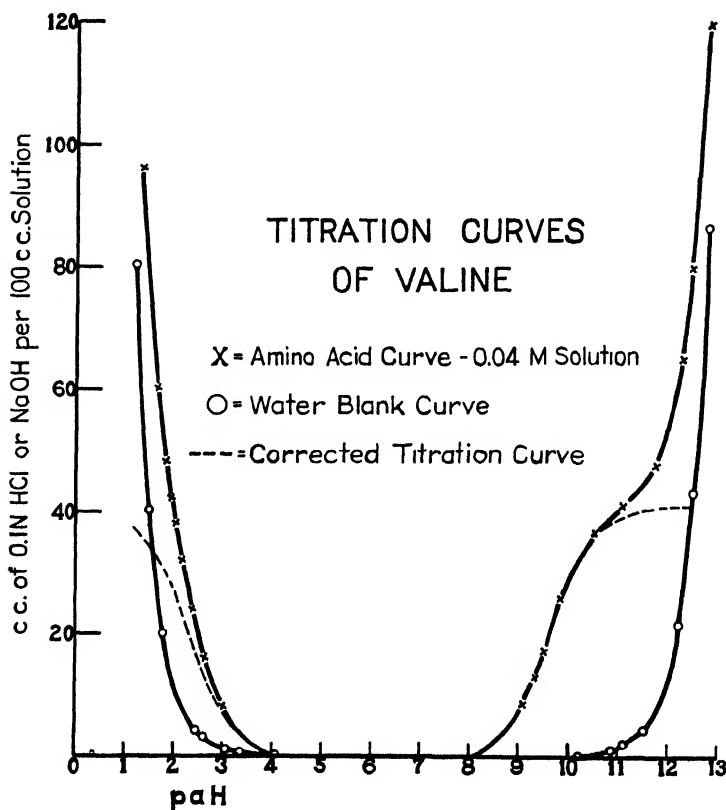
FIG. 4.

sents the apparent dissociation constant based on the assumption (1) that the concentrations of anion and of salt are equal, (2) that the remaining acid is totally undissociated, and (3) that the

concentrations of both constituents of the system are equal to their activities. Similarly in accord with the simple equation

$$pa_H = pK_w - pK'_b + \log \frac{base}{salt}$$

the reading of the point of half neutralization with acid would represent $pK_w - pK'_b$ or at that point

$$pK'_b = pK_w - pa_H$$

These equations obviously do not give the true values for pK'_a and pK'_b because (1) the concentration of salt is not equal to the concentration of anion (or cation) on account of hydrolysis, (2) the inherent errors in points close to the mid point of the curve have an unduly large effect on the position of the mid point. Moreover, the error of plotting is not entirely negligible.

For these reasons it seems preferable to calculate the dissociation constants directly from the data by means of a proper equation, rather than to read them from the titration mid points. This has the advantage of decreasing the error due to deviations of the individual points from their correct values. The following equation was used in calculating values for pK'_a.

$$pK'_a = pa_H + \log \frac{C_A - C_{Na^+} + C_{OH^-}}{C_{Na^+} - C_{OH^-}}$$

where C_A is the total amino acid concentration, C_{Na^+} and C_{OH^-} are the concentrations of sodium and hydroxyl ions, respectively, in mols per liter. In the case of the first acid dissociation constant of glutamic acid and β-hydroxyglutamic acid, hydrolysis becomes negligible, but the assumption that all the anion comes from the salt is incorrect. Correction is made for this by modifying the equation, obtaining

$$pK'_a = pa_H + \log \frac{C_A - C_{Na^+} - C_{H^+}}{C_{Na^+} + C_{H^+}}$$

Apparent basic dissociation constants may be calculated from the equation

$$pK'_b = pK_w - pa_H + \log \frac{C_A - C_{Cl^-} + C_{H^+}}{C_{Cl^-} - C_{H^+}}$$

where pK_w is equal to the reciprocal logarithm of the dissociation constant of water and is numerically equal to 13.998.[3]
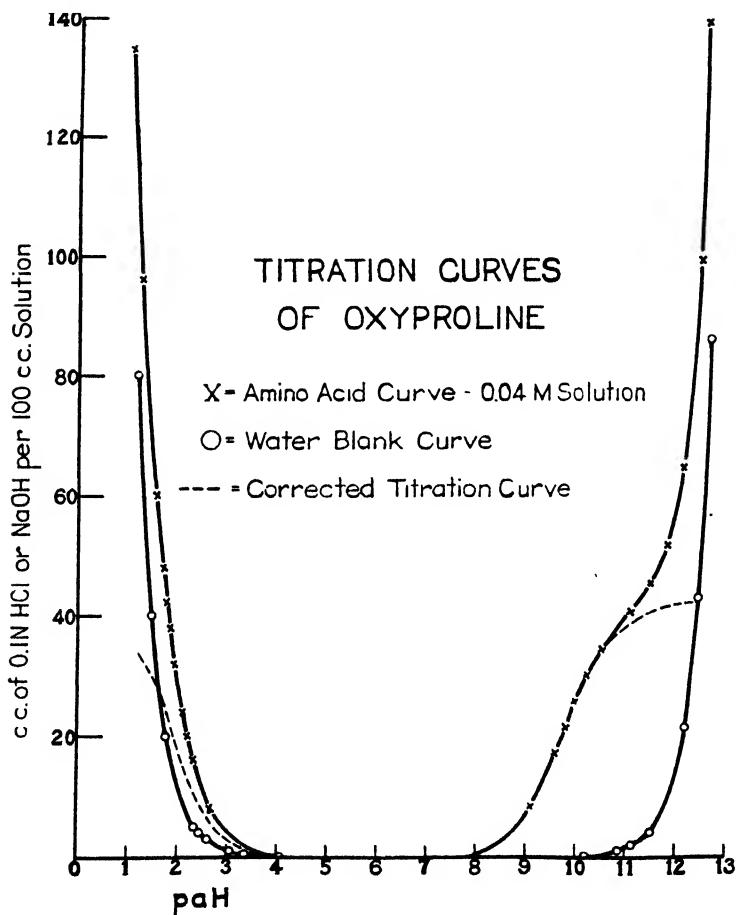

TITRATION CURVES
OF OXYPROLINE

X = Amino Acid Curve - 0.04 M Solution

O = Water Blank Curve

--- = Corrected Titration Curve

Fɪɢ. 5.

We are entirely aware of the inaccuracies of our equations due to equating a_H^+, the activity of the hydrogen ion, to C_H^+ the

[3] For the sake of conforming with recent usage (Lewis and Randall (19)) the value for K_w at 25° has been taken as 1.005×10^{-14}. We are aware that this value may need revision as better data become available.

concentration, and to using concentration rather than activities for the other constituents, notably the amino acid ions formed.
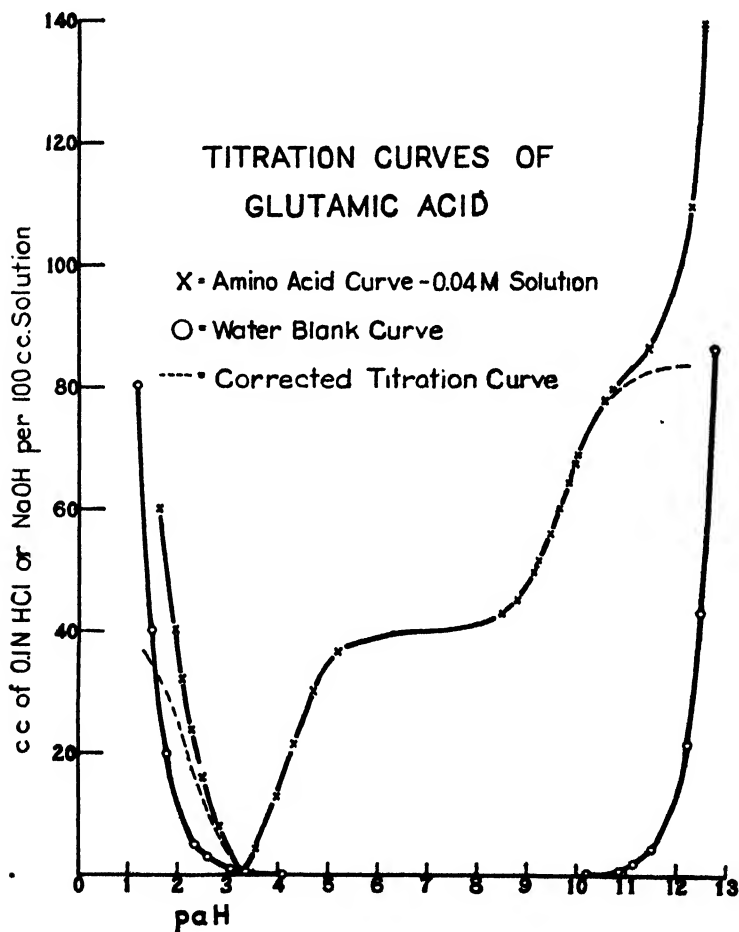
FIG. 6.

Since no data relating to the activity coefficients of the ions formed by amino acids are available, we find it necessary to assume them equal to 1, or to make some less simple assumption, *e.g.* that the value of the activity coefficient is the same for an

amino acid ion as for the acetate ion.   Calculation of pK was
made on the latter assumption, with activity coefficients which
have been deduced by Cohn, Heyroth, and Menkin (20) for the
acetate ion.   Moreover, we have used the value of unity for the

TITRATION CURVES
OF β-HYDROXY GLUTAMIC ACID

X: Amino Acid Curve-0.038 M Solution

O: Water Blank Curve

----: Corrected Titration Curve
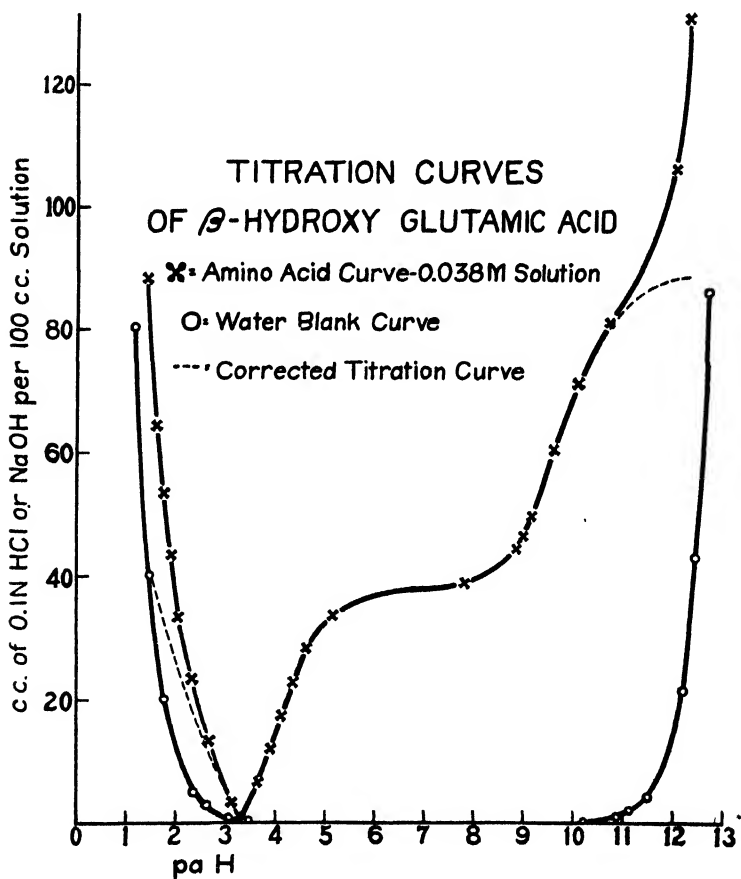
Fɪɢ. 7.

activity coefficient of the undissociated acid.   According to the
convention of Randall and Failey (21) this is permissible at zero
ionic strength, but due to the salting out effect, it is not correct as
appreciable amounts of strong acid or base are added.   With the

above assumptions, all values for pK_a were found to be slightly higher than for pK'_a, the magnitude being roughly 0.05 to 0.1 unit. Due to the fact that the values of pK_a thus calculated are less constant than those for pK'_a, we consider it preferable to record the latter values obtained by use of the simple incorrect assumption at present. We hope to recalculate the data as soon as values for the activity coefficients of the amino acids become available.[4]

Table I gives the summarized results of these calculations of the apparent dissociation constants.

TABLE I.

Apparent Dissociation Constants.[*]

	K'_a	K'_b	$pI = \frac{1}{2} (pK'_a + pK_w - pK'_b)$
Glutamic acid	5.62×10^{-5}	1.55×10^{-12}	3.22
	2.19×10^{-10}		
Serine	7.08×10^{-10}	1.62×10^{-12}	5.68
Valine	2.40×10^{-10}	2.09×10^{-12}	5.97
d-Isoleucine	2.09×10^{-10}	2.29×10^{-12}	6.02
Oxyproline	1.86×10^{-10}	8.32×10^{-13}	5.82
β-Hydroxyglutamic acid .	5.82×10^{-5}	2.12×10^{-12}	3.28
	2.76×10^{-10}		
Norleucine	1.72×10^{-10}	2.46×10^{-12}	6.08

* The nomenclature used is in accordance with the classical method. This was done solely for the sake of conforming with established usage and does not imply that the newer concept of ampholyte dissociation as given by Bjerrum (22) is not preferable.

It is seen from these results that the effect of the hydroxyl groups of serine, oxyproline, and β-hydroxyglutamic acid are to increase the magnitude of the acid dissociation, themselves having no appreciable power of combining with base. The fact that oxyproline has a basic dissociation, which is lower than an amino group, but still appreciable, ranks it with proline as a possible source of acid binding in the protein molecule.

The values for the isoelectric point, given in the fourth column of Table I, are simply those calculated from the well known equa-

[4] Work on the activity coefficients of amino acids is in progress.

tion independently of the obvious fact that for all the amino acids except glutamic acid and β-hydroxyglutamic acid, the isoelectric region is a wide zone, dissociation only taking place in appreciably acid or basic regions.

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THE DISSOCIATION CONSTANTS OF ORNITHINE.

By WERNER SCHMIDT, PAUL L. KIRK, and CARL L. A. SCHMIDT.

(From the Division of Biochemistry, University of California Medical School, Berkeley.)

Although ornithine is not usually considered as one of the amino acids, since apparently it is not found in the native protein molecule, it nevertheless possesses the characteristics usually associated with amino acids. On account of its biological importance, it was considered desirable to determine the dissociation constants.

Ornithine was prepared by hydrolysis of arginine with barium hydroxide and converted into ornithuric acid by treating with benzoyl chloride. This was repeatedly recrystallized from the acidified solution and finally hydrolyzed by boiling with hydrochloric acid. After extracting the benzoic acid with ether, the ornithine chloride was precipitated from the concentrated solution by treating with absolute alcohol. The final product was a mixture of ornithine mono- and dichloride. The substance was prepared in the laboratories of the Physiologisch-Chemisches Institut of the University of Würzburg.

The technique used in preparing the titration curve and the method employed in determining the dissociation constants are the same as described in the preceding paper. The titration curve (Fig. 1) shows that the substance is a strong base, as would be expected from its structure. The following values were calculated. $K'_a = 1.74 \times 10^{-11}$, $K'_{b_1} = 4.46 \times 10^{-6}$, $K'_{b_2} = 8.70 \times 10^{-13}$, pI = 9.70.
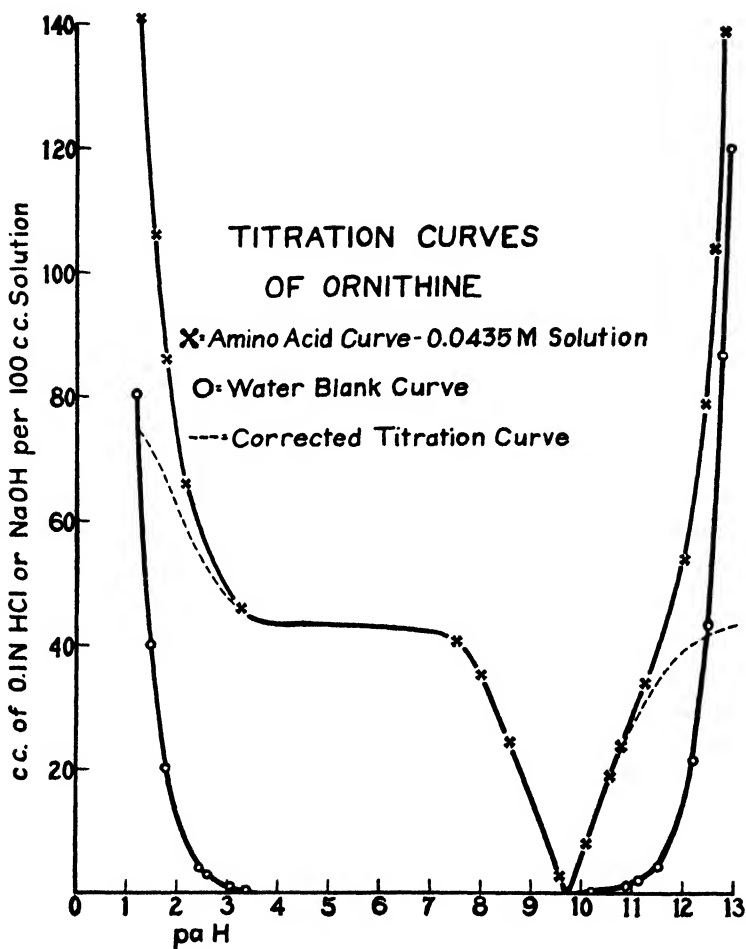
FIG. 1. Titration curve of ornithine.

BLOOD REGENERATION IN SEVERE ANEMIA.

XV. LIVER FRACTIONS AND POTENT FACTORS.

By WARREN M. SPERRY, C. A. ELDEN, FRIEDA S. ROBSCHEIT-ROBBINS, AND G. H. WHIPPLE.

(*From the Departments of Biochemistry and Pathology, The University of Rochester School of Medicine and Dentistry, Rochester, New York.*)

When it became apparent that liver feeding caused a great increase in red cell and hemoglobin production in standard anemic dogs it was logical to attempt a concentration of these factors which have this startling effect upon body manufacture of hemoglobin. From time to time we have made extracts and fractions utilizing not only liver but kidney and apricots. Whipple and Robscheit-Robbins (8) reported some experiments with simple fractions and residues to show that watery and alcoholic fractions both contain distinct potent factors. We (4) have recently shown that the inorganic ash of liver, kidney, and apricots contains some of the potent materials. This obviously indicates some significant salt effect.

A study of a liver fraction has been completed recently (5) which has a distinct bearing on these experiments tabulated below. This liver extract, No. 343 N. N. R., was manufactured by Eli Lilly and Company under the direction of the Committee on Pernicious Anemia of the Harvard Medical School according to the method of Cohn, Minot, Alles, and Salter (1). This extract is known to be potent in pernicious anemia but it is relatively inert in this type of experimental anemia. We may say that it retains 10 to 20 per cent of the original liver potency.

All this evidence makes it quite clear that in this type of simple secondary anemia there are several factors concerned in this liver effect. In human anemia as a rule we are dealing with a complex condition so that it is not unreasonable to suppose that several factors are concerned in the human anemia as well as in

the relatively simple experimental anemia. This makes the problem of isolation increasingly difficult but perhaps even more intriguing. It is possible too that this knowledge may eventually contribute to a better understanding of the complex internal protein and pigment metabolism.

Experimental Observations.

The general methods used in these anemia experiments have been reviewed briefly in this *Journal* recently (4) and given in detail in Papers I (6) and V (7) of this series. All fractions, equivalent to 200 to 600 gm. of raw liver, were carefully mixed with the basal bread ration—liquids and residues as such. If in paste form, the latter was first dissolved in a small volume of hot water and then added to the bread mixture. Occasionally it was necessary to resort to the administration of liquid extracts by stomach tube when food consumption was unsatisfactory.

We planned to make a number of simple aqueous and alcoholic extracts, varying the pH, temperature, acid or alkali, and time of extraction, until we found a method of isolating all or a large portion of the active material in one fraction to which various purification procedures could then be applied. Our experience so far has been that almost any method of fractionation will remove some of the potent factors.

A few experiments with a simple hydrochloric acid extract have been cited (8). Beef liver was boiled 2 minutes with 0.1 per cent HCl and filtered. This residue was then extracted with alcohol. The three fractions (aqueous extract, alcoholic extract, and residue) contained roughly each 20 to 25 per cent of the active material of whole liver with about a 25 per cent loss due to method. The moderate degree of success of this attempt encouraged us to make more HCl extracts, using more concentrated acid and longer periods of extraction. First it seemed desirable to obtain some information concerning the buffer power of minced liver and the filterability of liver mixtures at various pH's. To that end a rough titration curve was worked out. Small samples of minced liver were heated with 3 times their weight of HCl of various concentrations (Table 151) for 4 hours on the steam bath at 65–75°, then for 5 minutes at 80° over a gas flame, and filtered. The pH values of the filtrates were determined with the hydrogen electrode

after 20 minutes shaking. These values are uncorrected and probably not very accurate but they seem to have some relative value since, when plotted against the volume of normal acid per 100 gm. of liver (in terms of logarithms), they fall on a smooth curve with small variations. From this curve it may be calculated that the pH of the watery extract tested previously (8) was between 4.8 and 4.9 if we assume that the short period of boiling employed in making that extract was sufficient to allow the liver to exercise as much buffering power as in the longer heating periods at lower temperatures used in this experiment.

TABLE 151.

pH of Liver Extracts Made with Different Concentrations of HCl.

Sample No.	Weight.	Normality of HCl.	Volume of acid.	Volume of N acid per 100 gm. liver.	Log.	pH
	gm.		*cc.*	*cc.*		
1	100	1.0	300	300	2.477	0.28
2	106	0.75	318	225	2.352	0.40
3	101	0.5	303	150	2.176	0.64
4	107	0.25	321	75	1.874	1.12
5	129	0.1	386	30	1.477	2.95
6	125	0.05	374	15	1.176	4.15
7	128	0.04	384	12	1.080	4.35
8	126	0.03	378	9	0.954	4.77
9	122	0.02	366	6	0.777	5.25
10	125	0.01	375	3	0.477	5.65

A great difference in the ease of filtration was observed at the different pH's studied, with a sharp break at about 4.3; *i.e.*, just below the isoelectric point for the characteristic liver proteins. The first six extracts, at lower pH's, could be filtered through cheese-cloth only with difficulty; Extract 7 filtered readily through the cloth, while the others filtered rapidly through paper with increasing ease as the pH increased to 5.65. When alkali was added to Extracts 1 to 6 to bring the pH to about 5.0 there was a coagulation of proteins which could be filtered readily on paper.

Methods Employed in Preparing Liver Fractions.

Fractions are lettered in chronological order.   They are grouped according to the general method employed.   From 10 to 15 kilos of *raw beef liver*, minced to the point where it could be poured readily from one flask into another, were used in preparing each extract described.   Concentration of filtrates, except where otherwise noted, was accomplished by blowing a stream of air from an electric fan over the liquid in large evaporating dishes on a steam bath.   An ordinary cheap fruit-press was used for filtering several of the extracts as noted.   Some trouble was experienced due to

TABLE 152.

Hydrochloric Acid Fractions of Beef Liver and Hemoglobin Production.

Fraction.	Method.	Dog No.	Liver equiva-lent.	Feeding days.	Hb pro-duction, total gm.
			gm.		
Aqueous HCl.	Filtrate A-4.	24–49	600	10	30
	" "	24–45	600	9	12
	" RQ-8.	18–114	600	14	37
	" "	19–104	600	17	30
	Residue A-3.	24–26	600	10	18
	" "	21–67	600	8	13
	" RQ-5.	24–46	600	14	70
	" A-7.	24–45	600	14	107
Alcoholic HCl.	Filtrate B-1.	24–59	400	13	41
	" "	24–49	300	16	68
	" E-7.	24–22	400	14	25

the breaking of the heavy, unbleached muslin when forced out between the slats of the filtering barrel.   This trouble was overcome in part by fastening a band of steel about the barrel.

Fraction A.—Liver was heated at 70–80° for 2 to 3 hours with 3 volumes of 0.16 N HCl, which, from the above mentioned curve, should give a final pH of about 2.   The mixture was filtered through cheese-cloth with great difficulty, as expected from the preliminary experiments above.   The residue, A–3, was heated for 2 hours at 70° with 0.01 N HCl, filtered, and dried.   Residue A–3 fed to standard dogs caused an increased hemoglobin production of 18 and 13 gm. (Table 152).   Enough

NaOH determined by preliminary tests was added to the combined filtrates to make the pH about 4.6, the coagulated proteins were filtered, and Filtrate A–4 was concentrated. The concentrated filtrate, A–4, fed to standard dogs caused an increased hemoglobin production of 30 and 12 gm. (Table 152). The proteins, A–7, from this last filtration were washed with 0.1 N sodium acid phosphate and dried. The protein residue, A–7, fed to a standard dog caused an increased hemoglobin· production of 107 gm. (Table 152).

Fraction RQ was prepared essentially as described in a previous publication (8). Liver was boiled for 3 minutes with 3 weights of 0.1 per cent HCl and filtered, first through gauze, then paper. The filtrate, RQ–8, was neutralized to litmus with NaOH and concentrated. Filtrate RQ–8 fed to standard dogs caused an increased hemoglobin production of 37 and 30 gm. (Table 152). The residue, RQ–5, after extraction with 95 per cent alcohol was dried and freed of alcohol before feeding. Residue RQ–5 fed to a standard dog caused an increased hemoglobin production of 70 gm. (Table 152).

Fraction B.—Liver was heated at 70° for 3 hours with 3 volumes of 0.2 N alcoholic HCl and filtered through paper. Preliminary experiments had shown that such a filtration was possible though slow. The residue was washed with 0.02 N alcoholic HCl and filtered in the fruit-press. The washings were combined with the first filtrate and concentrated until the alcohol was removed completely. This filtrate, B–1, was fed to two standard dogs and caused an increased hemoglobin production of 41 and 68 gm. (Table 152). See also Table 156 for details.

Fraction E was prepared in essentially the same manner as Fraction B except for the use of 0.1 N alcoholic HCl in washing and the addition of 0.1 N NaOH to the filtrate (1 volume to 2 volumes of filtrate). The resulting precipitate was removed by filtration with paper, and the filtrate, E–7, which gave a negative biuret reaction, was concentrated. When fed to a standard dog Filtrate E–7 caused an increased hemoglobin production of 25 gm. (Table 152).

Fraction F.—Liver was heated at 70° for 6 hours with 4 volumes of H_2SO_4, 5 per cent by volume, and filtered through paper. The

residue, F–4, was washed with water to free it from acid and dried. Residue F–4 fed to a standard dog caused an increased hemoglobin production of 116 gm. (Table 153). The combined washings and filtrate, F–6, were adjusted to pH 3.2 with barium hydroxide, filtered, and concentrated. Filtrate F–6, fed to two standard dogs, caused an increased hemoglobin production of 60 and 50 gm. (Table 153). See also Table 157 for details.

Fraction N.—Since Residue F–4 was so potent another sulfuric acid extract was made, the residue corresponding to F–4 being extracted three times at 39° with 5 per cent H_2SO_4 for 10 to

TABLE 153.

Sulfuric Acid Fractions of Beef Liver and Hemoglobin Production.

Fraction.	Method.	Dog No.	Liver equivalent.	Feeding days.	Hb production, total gm.
			gm.		
H_2SO_4	Filtrate F-6.	24–49	600	14	60
	" "	21–67	300	14	50
	Residue F-4.	24–59	400	14	116
H_2SO_4	Filtrate N-2.	25–24	200	21	50
	" N-1.	21–67	200	14	50
	Residue N-11.	24–45	600	14	67
H_2SO_4	Filtrate P-4.	24–59	400	14	24
	Residue P.	24–49	500	14	84
	" " ash.	23–3	500	14	24
H_2SO_4	" AH.	23–1	500	14	34
	Precipitate AH.	24–90	500	14	35
	" " ash.	24–90	500	14	28

12 hours. The resulting residue, N–11, was washed with water and dried. Residue N–11 fed to a standard dog caused an increased hemoglobin production of 67 gm. (Table 153). The combined filtrates and washings were divided equally; N–1 was adjusted to pH 5.5 and N–2 to 8.3 with $Ca(OH)_2$. After filtering they were concentrated. Filtrates N–1 and N–2 fed to standard dogs each caused an increased hemoglobin production of 50 gm. (Table 153).

Fraction P was made like Fraction N except that the combined washings and filtrate, P–4, were concentrated to a small volume and then adjusted to pH 4 with $Ca(OH)_2$. Filtrate P–4 fed to a

standard dog caused an increased hemoglobin production of 24 gm.
(Table 153).   The residue, P, left after extraction with H_2SO_4,
was washed with water until the pH of the washings was 2.5.
Residue P fed to a standard dog caused an increased hemoglobin
production of 84 gm. (Table 153).   Since Residue P was active
after this treatment a portion was ashed.   Residue P ash fed to a
standard dog caused an increased hemoglobin production of 24 gm.
(Table 153).   •

Fraction AH.—This sulfuric acid extract was made as nearly
as possible according to the last published directions of Cohn,

TABLE 154.

Sodium Hydroxide Fractions of Beef Liver and Hemoglobin Production.

Fraction.	Method.	Dog No.	Liver equiva-lent.	Feeding days.	Hb pro-duction, total gm.
			gm.		
NaOH	Filtrate C-5.	21–67	400	14	25
	" "	24–25	300	14	15
	" C-9.	24–45	400	14	60
	Precipitate C-10.	21–67	400	14	25
NaOH	Filtrate K-1.	24–26	400	10	15
	" L-9.	25–24	400	14	22
	Precipitate L-8.	23–1	500	14	31
NaOH	" M-2.	24–49	280	14	44
	Residue AG.	24–45	500	14	50
	Precipitate AG.	24–45	500	14	50
	Alcoholic residue AG.	24–2	500	14	28

Minot, Alles, and Salter (1).   To each kilo of minced liver were
added 1 liter of water and 40 cc. of N H_2SO_4.   This mixture was
stirred frequently during a period of about 1 hour.   The pH was 5.
Water was added and after thorough mixing and settling, the
supernatant liquid was decanted.   The liver residue was washed
approximately twenty times by adding fresh portions of water,
mixing, allowing to settle, and decanting the supernatant liquid,
which, in the final washing was almost colorless.   The amount of
filtrate and washings was 250 liters for 14.6 kilos of liver.   These
washings were heated to 70°, filtered through paper to remove the
heat-coagulated proteins, and stored in the ice box to prevent

spoiling. The clear, light amber-colored liquid was concentrated *in vacuo* to 1 liter.   To prevent frothing a small amount of toluene was added.   This concentrate was poured slowly into sufficient alcohol to make the resulting solution approximately 95 per cent alcohol by volume.   The resulting precipitate was filtered off, dried, and washed with ether.   It weighed 300 gm. Half was fed as such.   Precipitate AH fed to a standard dog caused an increased hemoglobin production of 35 gm. (Table 153). The other half was ashed.   54 gm. of ash were obtained from 150 gm. of precipitate.   Precipitate AH ash fed to a standard dog caused an increased hemoglobin production of 28 gm. (Table 153). Residue AH fed to a standard dog caused an increased hemoglobin production of 34 gm.   This was the original liver residue after being treated with diluted H_2SO_4 and washed thoroughly with water.

Fraction C.—Liver was heated at 85–90° for 4 to 5 hours with 4 times its weight of 0.1 N NaOH and filtered in the fruit-press with considerable difficulty.   The filtrate was adjusted to pH 4.6 with HCl, the coagulated proteins resulting were filtered on paper, and the filtrate, C–5, concentrated.   Filtrate C–5 fed to two standard dogs caused an increased hemoglobin production of 25 and 15 gm. (Table 154).   The coagulated proteins were not fed.   The gelatinous residue from the first filtration was reminced and an attempt was made to reextract with 2 per cent NaOH as before.   The resulting mixture could be filtered only with difficulty and finally the whole was brought to pH 4.6 with HCl.   The resulting curdy solids, C–10, now filtered readily on paper.   The filtrate, C–9, was concentrated and the solids, C–10, were dried.   Filtrate C–9 fed to a standard dog caused an increased hemoglobin production of 60 gm.   Precipitate C–10 fed to a standard dog caused an increased hemoglobin production of 25 gm. (Table 154).

Fraction K was made as nearly as possible according to the first published directions of Cohn, Minot, Fulton, Ulrichs, Sargent, Weare, and Murphy (2).   Liver was shaken for 1½ hours with 1.7 times its weight of 0.3 N NaOH, when it coagulated to a gelatinous mass.   The pH was brought to 9 with difficulty by careful addition of 0.5 N HCl and the mixture filtered with great difficulty in the fruit-press.   The filtrate was adjusted to pH 5.2

with HCl and the precipitated solids were filtered on paper.   The filtrate was heated to 70° and the coagulated proteins filtered as before.   The resulting filtrate, K–1, was concentrated.   Filtrate K–1 fed to a standard dog caused an increased hemoglobin production of 15 gm. (Table 154).

Because of the great difficulty experienced in filtering alkaline extracts and in adjusting the pH, experiments were carried out in an attempt to obtain information concerning these points and
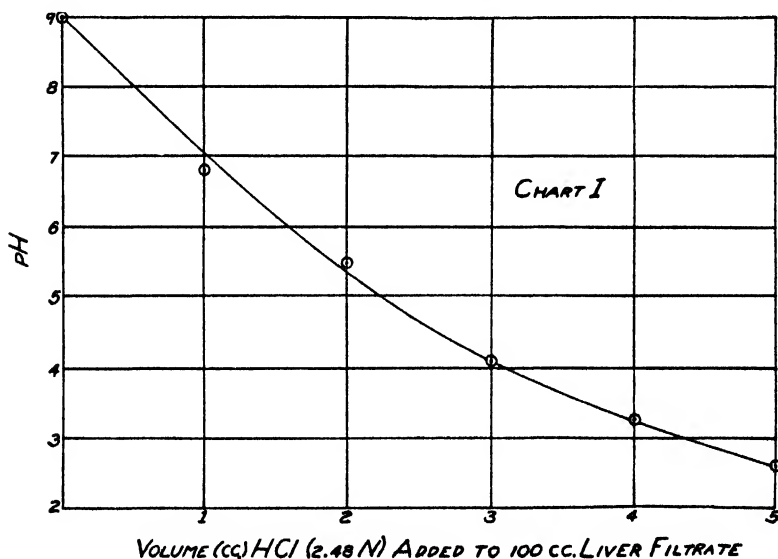

CHART I.

arrive at a satisfactory procedure.   In the first series the effect of volume and amount of alkali on the pH and viscosity was studied.   It was found that 3 volumes of 0.05 N NaOH per volume of liver gave a pH of 9 and an optimum viscosity of 1.5 to 2.5.   In the second series 0.05 N NaOH was added to portions of liver in this proportion and allowed to stand for varying periods of time. It was noted that there was a gradual but insignificant decrease of pH of 0.2 over a period of 1 hour.   When allowed to stand overnight, however, at room temperature the pH reached 5.   In the

third series different amounts of relatively strong standard HCl (2.48 N) were added to 100 cc. portions of the filtrate obtained from the treatment of 1 part of liver with 3 volumes of 0.05 N NaOH. Relatively strong acid was used in order to reduce volume changes to a minimum. The results are indicated graphically in Chart I. By using this information Fractions L, M, and AG were prepared.

Fraction L.—Liver was stirred with 3 volumes of 0.05 N NaOH for about 20 minutes and filtered in the fruit-press. By reference to Chart I sufficient HCl was added to the filtrate to adjust it to pH 5 and the precipitated proteins (Precipitate L–8) were filtered on paper. Precipitate L–8 fed to a standard dog caused an increased hemoglobin production of 31 gm. (Table 154). The resulting filtrates were heated to 70° until all coagulable proteins separated and they were filtered on paper. The filtrate, L–9, corresponds to Fraction D of Cohn, Minot, Fulton, Ulrichs, Sargent, Weare, and Murphy (2). Filtrate L–9 fed to a standard dog caused an increased hemoglobin production of 22 gm. (Table 154).

Fraction M.—This fraction was made exactly like Fraction L except that the final filtrate corresponding to L–9 was evaporated to dryness on a water bath, made uniformly semisolid with a small amount of water, and the calculated amount of absolute alcohol added to bring the resulting solution up to 95 per cent. The precipitate obtained was filtered off. Precipitate M–2 fed to a standard dog caused an increased hemoglobin production of 44 gm. (Table 154).

Fraction AG.—This fraction was made essentially like Fractions L and M with the exception that the liver residue was washed with water as described under Fraction AH until the washings were almost colorless. The combined NaOH extract and washings from 12.6 kilos of liver, which had a total volume of about 200 liters, were heated to 70°, filtered through paper, and concentrated *in vacuo* as in Fraction AH. The concentrate was treated with sufficient absolute alcohol to bring the resulting solution up to 95 per cent and filtered. The precipitate, AG, remaining, was dried and washed with ether. 10.7 gm. of this precipitate were equivalent to 500 gm. of fresh liver. It contained 35 per cent ash. Precipitate AG fed to a standard dog caused an increased hemoglobin production of 50 gm. The alcoholic filtrate was concentrated to one-third its volume when a material insoluble in alcohol

precipitated out (Alcoholic Residue AG). It was washed with 95 per cent alcohol and dried. 1.1 gm. of this material were equivalent to 500 gm. of fresh liver. Alcoholic Residue AG fed to a standard dog caused an increased hemoglobin production of 28 gm. (Table 154). Residue AG was the original liver residue left after being treated with dilute NaOH and washed thoroughly with water. Residue AG fed to a standard dog caused an increased hemoglobin production of 50 gm. (Table 154).

Extract S was made by treating liver with 3 volumes of a saturated solution of Ca(OH)₂ for about 20 minutes, adjusting the pH to 8 with HCl, and filtering. The residue, S–3, was reextracted six

TABLE 155.

Digests and Miscellaneous Fractions of Beef Liver.

Fraction.	Method.	Dog No.	Liver equivalent.	Feeding days.	Hb production, total gm.
			gm.		
Ca(OH)₂	Filtrate S-9.	23–1	500	14	17
" "	" "	24–26	500	14	13
	Residue S-3.	24–25	500	14	32
Pancreatic digest.	Filtrate D-3.	24–46	235	14	33
	Residue D-4.	24–59	235	14	42
Pepsin digest.	Filtrate J-3.	24–26	270	14	53
Autolysis.	" G-3.	25–24	400	14	14
Picric acid.	Crystals H.	24–26	600	14	9

times with an equal volume of Ca(OH)₂ solution and dried. Residue S–3 fed to a standard dog caused an increased hemoglobin production of 32 gm. (Table 155). All the filtrates were combined, adjusted to pH 5 with HCl, and filtered. The filtrate, S–9, concentrated and fed to standard dogs, caused an increased hemoglobin production of 17 and 13 gm. (Table 155).

Digest D was made by digesting liver with 2 gm. of pancreatin per 100 gm. of liver in a slightly alkaline medium at 39° for 18 days. From time to time pH determinations were made and the necessary NaOH was added to keep the mixture alkaline. Chloroform and toluene were added as preservatives and at the end of digestion the mixture was filtered. The filtrate, D–3, was concentrated

and the residue, D–4, was dried.   Filtrate D–3 fed to a standard dog caused an increased hemoglobin production of 33 gm.   Residue D–4 fed to a standard dog caused an increased hemoglobin production of 42 gm. (Table 155).

TABLE 156.

Alcoholic Hydrochloric Acid Fraction B-1 Contains Potent Factors for Hemoglobin Regeneration.

Diet periods 1 wk. each. Food, gm. per day.	Food consumed.	Wt.	Plasma vol.	RBC	Color index.	Hb index.	RBC hematocrit.	Blood Hb level.	Hb removed, bled.
	per cent	*kg.*	*cc.*	*million*			*per cent*	*per cent*	*gm.*
Br. 400, salm. 100	100	16 2	982	4,6	0 50	1.83	25.2	46	1.4
" 400, " 100 ...	100	16 6	1051	4,6	0 48	1 86	23 7	44	1.3
B-1* equiv. 300.......	89	16.1	882	4,8	0 51	1.90	25.8	49	1.4
B-1* " 300.	92	15.9	871	5,3	0 51	2 01	21.6	43	31.1
Br. 350, salm. 100	100	16.8	1030	4,2	0 51	2.01	21 6	43	29.9
" 350, " 100....	100	16 5	956	4,7	0 54	1 99	21 9	44	13.2
" 350, " 100....	100	16 7	955	4,1	0 50	1.96	.21 1	41	1.2

Dog 24–49, bull, female, adult.

Dog 24–59, bull, male, young adult.

Br. 500, salm. 100	100	17.2	1040	6,0	0.41	1.88	26.2	49	1.5
B-1† equiv. 600.......	94	16 8	934	6,4	0.48	1 82	27.4	50	20 2
B-1† " 500.......	100	17.3	945	6,8	0.45	1.86	23.4	44	26.8
Br. 450, salm. 100	100	16.9	1024	5,7	0.44	1.85	21.2	39	13.2
" 500, " 100....	100	17 3	988	5,1	0.39	1 79	22.4	40	1.2

* B-1 supplemented by bread 350 gm., salmon 100 gm., daily.

† B-1 supplemented by bread 400 gm., salmon 75 gm., daily.

Digest J was made by digesting liver with pepsin (0.5 gm. per 100 gm. of liver) in an incubator at 39° for 14 days.   2 volumes of 0.1 N HCl were added at the start and pH determinations were made from time to time, the necessary HCl being added to keep the reaction at about pH 3.   Chloroform was used as a preservative.   The mixture was filtered at the end of the digestion period

and the filtrate, J–3, after being concentrated was adjusted to pH 5.4. Filtrate J–3 fed to a standard dog caused an increased hemoglobin production of 53 gm. (Table 155).

Digest G.—Liver was allowed to autolyze at 39° with the proper preservatives for 16 days. The mixture was filtered and the filtrate, G–3, the pH of which was 4.75, was concentrated. Filtrate G–3 fed to a standard dog caused an increased hemoglobin production of 14 gm. (Table 155).

Fraction H.—4.5 gm. of a white crystalline product were prepared from 7900 gm. of liver as insulin is prepared from pancreas, according to the picric acid method of Dodds and Dickens (3). The preparation was tested for blood sugar reduction on rabbits by Dr. du Vigneaud and proved to be negative. This fraction, Crystals H, fed to a standard dog caused an increased hemoglobin production of 9 gm. (Table 155).

Table 156 gives the complete experimental data on two dogs for two complete experiments dealing with an alcoholic HCl extract of beef liver (B–1). The total hemoglobin production only is given in Table 152. All other figures for total hemoglobin production in Tables 152 to 155 inclusive are taken from complete experiments as are detailed in Table 156. These experimental periods are taken from the experimental history which is continuous for each dog. In these standard dogs the control periods alternate with special diet periods as indicated but the anemia is continuous at one-third normal and the stimulus for new red cell and hemoglobin production presumably constant and maximal.

This particular alcoholic hydrochloric acid liver fraction, B–1, Table 156, contains a large amount of the potent factors found in whole liver, probably from 50 to 60 per cent total potency. For this reason it deserves further study. Whether it contains factors potent in other types of anemia remains to be seen.

Table 157, like Table 156, gives complete experimental data on two dogs fed a potent sulfuric acid watery extract of beef liver (F–6). The total hemoglobin production only is given in Table 153. This fraction contains probably more than half the potent factors in whole liver as they are measured in this type of experimental anemia. For this reason this type of extract deserves further study.

We note that these extracts at times cause some impairment of appetite.   Also there is a tendency for the plasma volume to show a distinct shrinkage.   This is probably to be explained by a salt effect and has been 'frequently noted in heavy salt mixture feed-

TABLE 157.

Sulfuric Acid Fraction F-6 Contains Potent Factors for Hemoglobin Regeneration.

Diet periods 1 wk. each. Food, gm. per day.	Food consumed.	Wt.	Plasma vol.	RBC	Color index.	Hb index.	RBC hematocrit.	Blood Hb level.	Hb removed, bled.
	per cent	kg.	cc.	million			per cent	per cent	gm.
Dog 24–49, bull, female, adult.									
Br. 400, salm. 100	100	16.7	955	4,1	0 50	1 96	21.1	41	1 2
F-6* equiv. 600	88	16 8	988	4,3	0 55	1.91	24 8	47	1.4
F-6* " 600.......	67	16 2	861	5,8	0 59	2.11	26.2	55	35.7
Br. 350, salm. 100 ...	97	16 6	1005	4,5	0.58	2 00	23.7	48	13 9
" 350, " 100	100	16.3	956	4,7	0 56	2.08	23 7	49	14 4
" 350, " 100	100	15 7	908	4,6	0.51	2 03	23.0	47	1.3
Dog 21–67, bull, male, adult.									
Br. 225, salm. 100, Kl. 25.................	100	12 3	654	4,1	0.42	2.00	17.2	34	1.1
F-6† equiv. 300.......	100	12 3	584	5,7	0 53	1.85	21.7	46	15 9
F-6† " 300.......	87	12.6	615	5,0	0.60	2.14	24.8	53	17.7
Br. 250, salm. 100, Kl. 25.................	87	11.9	610	3,8	0.66	2.10	21.6	45	11.1
" "	100	12.1	628	4,4	0.54	1.95	24.3	47	1.3

* F-6 supplemented by bread 350 gm., salmon 100 gm., daily.

† F-6 supplemented by bread 225 gm., salmon 100 gm., Klim 25 gm., daily.

ing.   There is no constant change in the hemoglobin index which gives information as to the saturation of the red cell stroma by hemoglobin.

SUMMARY.

Practically all fractions and residues from beef liver described in this paper contain materials which the anemic dog is able to utilize to form new red cells and hemoglobin.   In spite of every endeavor the amount of potent material found in the various residues is consistently large.

Alcoholic hydrochloric acid and watery sulfuric acid fractions may contain more than half the original potency of whole liver as tested in standard dogs.   These fractions deserve further study.

Various digests made by pancreatic or peptic ferments or by sterile autolysis contain no unusual concentration of the potent factors.   The peptic digest contains the highest concentration of the potent factors and the autolytic digest the lowest.

Some alkaline extracts retain a large amount of the potency of whole liver.

A considerable salt effect is demonstrable in watery and alcoholic extracts as well as in residues.   It is not possible to say which salts are wholly responsible but in this type of anemia *iron* is of more importance than any other inorganic element.

All this evidence makes it more certain that a *number of factors* (organic and inorganic) are concerned in the *liver effect* as studied in simple experimental anemia due to bleeding.

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THE URINE OF THE GOOSEFISH (LOPHIUS PISCATORIUS): ITS NITROGENOUS CONSTITUENTS WITH SPECIAL REFERENCE TO THE PRESENCE IN IT OF TRIMETHYLAMINE OXIDE.

By ARTHUR GROLLMAN.

(From the Laboratory of Physiology, the Johns Hopkins University, School of Medicine, Baltimore.)

The analysis of the urine of *Lophius piscatorius*—variously called the monk-, goose-, frog-, or bellows-fish—has been recorded by several previous investigators (5, 9, 20, 23). Recent work (20) on renal function, as elucidated by studies on the kidney of this fish, has created particular interest in the composition of its urine. The prime aim of the present investigation was an attempt to determine the nature of the nitrogenous constituents. A number of analyses, previously reported by Professor E. K. Marshall, Jr., from this laboratory (20), showed that about one-half of the nitrogen excreted in the urine was present in an undetermined form, the usual analyses for ammonia, urea, creatine, uric acid, and creatinine accounting for only about 50 per cent of the total nitrogen present in the urine. Denis (5), as a result of her analyses of the urine of *Lophius*, had also noted this existence of a great part of the urinary nitrogen in an undetermined form.

In preliminary tests to determine the form in which this nitrogen existed, search was made for the presence of such compounds as the amino acids, purines, allantoin, betaine, *etc.*, which are common constituents of the urine of other animal species. Although the first of these compounds was found to be present in relatively large amount, the others were lacking or present in only slight traces. There still remained undetermined a large fraction of the total nitrogen. After several unsuccessful attempts to isolate this substance, it was found that, under certain conditions, rela-

tively large quantities of trimethylamine could be obtained. This amine was not originally present in the urine and it could not have been formed from any of the known constituents. A clue was thus furnished concerning the nature of the undetermined nitrogen. Further experimentation demonstrated that this undetermined nitrogen was present as trimethylamine oxide.

Sources of Materials.

The present investigation was carried out on urine obtained from various sources. The specimen indicated in Table I as Woods Hole was collected and sent to the author by Professor E. K. Marshall, Jr. This urine was obtained from fish caught in

TABLE I.

Distribution of Nitrogenous Constituents of Urine of the Goosefish
(Lophius piscatorius).

	Woods Hole		Atlantic I		Atlantic II.		Aquarium.	
	mg per 100 cc	*per cent of total N*	*mg per 100 cc.*	*per cent of total N*	*mg. per 100 cc.*	*per cent of total N*	*mg per 100 cc.*	*per cent of total N*
Total N.	180 0		373 0		337 0		56 0	
Ammonia N.........	1 0	0 5	1 3	0 4	1 0	0 3	0.7	1.3
Urea N.	1 2	0 7	0 4	0 1	0 6	0 2	1.5	2 7
Uric acid N.........	0 8	0 4	0 9	0 2	0 9	0 3	Trace.	
Amino acid N.......	25 8	14 3	27 0	7 2	18 0	5 3	4.5	8 0
Creatine N.	50 2	27 9	230 0	61.7	156.0	46 3	12 9	23 0
Creatinine N........	7 3	4 1	8 0	2 1	8.0	2.4	0 9	1 5
Undetermined N....	93 7	52 1	105 4	28 2	152.5	45.2	35 5	63 4

traps and brought to the Marine Biological Laboratory at Woods Hole where the urine was collected. The specimens indicated in Tables I and II as Atlantic I and Atlantic II were obtained on the George's Banks fishing grounds of the Atlantic ocean. This urine was collected from the bladders of fish freshly caught, and brought to the surface by a steam beam trawler. The author is indebted to Messrs. Clarence Birdseye and D. A. Read of the General Foods Company, Gloucester, Massachusetts, and to the captain and crew of the trawler, *Princeton,* for their kind cooperation in allowing him the facilities for collecting this material. The specimen indicated in Tables I and II as Aquarium was kindly

furnished by Professor Homer W. Smith, through the courtesy of Dr. C. M. Breder. This urine was obtained from fish collected by the New York Aquarium and kept by this institution during the period in which the urine was collected.

To all of those mentioned, the author wishes to express his thanks for their kind cooperation.

To prevent decomposition, sulfuric acid, toluene, or chloroform was added to the urine immediately after collection. As long as the urine maintained its original *acid* reaction no decomposition was obvious. However, as will be shown later, slow decomposition of the trimethylamine oxide, later found to be present in the urine, always occurred. In the case of the urine indicated as Atlantic I, this decomposition was complete due to the *alkalinity* of this specimen, as will be discussed later.

Total Nitrogen.

Analyses of the nitrogenous constituents of four specimens of the urine of *Lophius piscatorius* are given in Table I. These urines obtained from various sources, show a great variation in the total nitrogen concentrations, determined by the usual macro-Kjeldahl procedure. The urines obtained from the bladders of the freshly caught fish of the Atlantic show a total nitrogen content which is much higher than that heretofore reported.

Ammonia.

The ammonia determinations were made by absorbing on permutit and determining the absorbed ammonia by Nessler's reagent, according to the procedure of Folin and Bell (11). The values thus obtained agree with the results of Marshall and Grafflin (20), and are seen to be very small as compared to those obtained by others (5, 9). If the ammonia is determined by aeration, or by absorption on permutit with subsequent aeration, much higher values are obtained. This is due to the fact that trimethylamine is also absorbed by permutit. On aeration this amine is determined with the ammonia and renders the values of the latter too high. The source of the trimethylamine in *Lophius* urine will be discussed later.

In this connection, it may be stated that permutit does not absorb trimethylamine quantitatively as one would be led to

believe from the results of Whitehorn (28). Experiments with a pure solution of trimethylamine showed that only about one-third of the substance is taken up from dilute aqueous solution by permutit. Nessler's reagent does not form a soluble colored compound with trimethylamine, and hence may be used for determining ammonia in the presence of trimethylamine.[1]

The high ammonia results recorded by other investigators (5, 9) are due, no doubt, to their methods which were not specific for ammonia.

Urea and Uric Acid.

The urea values like the ammonia values are seen to be low as compared with those of all other observers (5, 9), except Marshall and Grafflin (20). The reason for this also lies, it is felt, in either the non-specificity of the methods employed or in a breakdown of other constituents by the vigorous reagents used in the older methods for determining urea. The present determinations were carried out by the urease method, with the procedure of Van Slyke (27). In this method one measures the carbon dioxide liberated by the action of urease on the urea. If one determines the ammonia liberated, it is necessary to do so by means of Nessler's reagent. Liberation of the ammonia by aeration, with its subsequent determination by titration, leads to the same errors encountered in the determination of urinary ammonia, as described above.

The low values of the urea nitrogen both in the urine and blood (20) are noteworthy and indicate that there is either a profound difference in the nitrogenous metabolism of this fish as compared to the warm-blooded vertebrates or else that the urea is excreted by some other channel than the kidneys.

Only traces of uric acid, as determined by the colorimetric method of Benedict and Franke (4), were found in the urine of *Lophius.*

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[1] The possible influence of the trimethylamine present in the urine of Lophius on the ammonia color development with Nessler's reagent, was investigated. In the presence of large amounts of di- or trimethylamine, there is precipitate formation with marked interference in the color development. In the concentration ranges encountered in this work, however, no error was detectable. Moreover, extraction of the chlorides of the total volatile base, by alcohol, gave results identical with those reported in this paper.

Amino Acids.

The amino acid nitrogen was determined by the colorimetric method of Folin (10) as well as by the nitrous acid method of Van Slyke (26). The two methods give concordant results. Unfortunately, both methods give only partial results with certain amino acids; hence, final values would necessitate actual isolation and identification of the amino acids present. About one-half of the total amino acids present was found to be soluble in ethyl alcohol.

The amino acid content of the urine of *Lophius* is of the same order of magnitude as the amino acid content of the blood of it and other fish, as previously found by other observers (6).

Creatine and Creatinine.

Creatine and creatinine were determined by the Folin-Benedict and Folin methods respectively (12). The high creatine values are noteworthy, in the fact that a third to over a half of the total nitrogen is excreted as creatine. That this material is actually creatine and not one of the many substances (15) which after the treatment with hydrochloric acid give the Jaffe color reaction, was demonstrated by isolating the material from urine according to the method described by Ackermann (1), converting a weighed quantity of the creatine thus obtained to creatinine picrate (8), and identifying the latter.

From 100 cc. of Atlantic II urine was isolated 0.48 gm. of creatine plus creatinine. This was about 90 per cent of the total amount of these substances present in the urine, as determined by the colorimetric method. 33.7 mg. of the above obtained material, treated according to the Folin-Benedict method (12) and compared colorimetrically with a known standard, gave 31.2 mg. of creatine. Another portion of 213.5 mg. was similarly treated with hydrochloric acid, and the creatinine formed was converted to the picrate. After recrystallization, 85 per cent of the theoretical yield of creatinine picrate was obtained. It melted at 205.0°. N found, 24.50 per cent; N calculated for $C_4H_7ON_3 \cdot C_6H_3O_7N_3$, 24.56 per cent.

The relative amounts of creatine and creatinine in the urine of *Lophius* resemble that found in the case of reptiles and birds (16).

As to the source of the creatine, nothing can be stated except that it is probably a catabolic product of the large amounts of protein which *Lophius* ingests. Its possible derivation from choline, betaine, cystine, histidine, guanidine, or the other substances suggested as precursors of creatine in the body, now remains purely conjectural.

Trimethylamine Oxide.

Trimethylamine oxide was found to be present in the urine of *Lophius* in high concentration. In order to isolate this substance for identification, the urine was evaporated to dryness on the water bath in the presence of HCl, and the residue extracted with absolute alcohol. The alcohol was driven off on the water bath leaving a brownish, syrupy liquid containing the trimethylamine oxide together with some of the other nitrogenous substances of the urine. It may be separated from these substances according to the procedures of Suwa (24) or Poller and Linneweh (22). In their method the syrupy liquid is dissolved in 5 per cent sulfuric acid, precipitated by saturated phosphotungstic acid in 5 per cent sulfuric acid solution, allowed to stand overnight, and the precipitate removed on a Buchner funnel and washed with 5 per cent sulfuric acid. This precipitate is dissolved in acetone and decomposed with baryta. After removal of the excess of baryta with carbon dioxide, the solution is evaporated to a syrup and the trimethylamine oxide precipitated as the aurichloride with 30 per cent aqueous gold chloride. In subsequent work it was found that the trimethylamine oxide, when only slightly contaminated with other substances, may be precipitated directly from the syrupy liquid obtained after evaporating the alcohol as described above. After recrystallization the comparatively pure compound was obtained.

The aurichloride of trimethylamine oxide was obtained from each of the urines, except Atlantic I, in which, for reasons to be described later, the oxide had been completely decomposed to trimethylamine. A typical experiment follows.

150 cc. of Atlantic II urine were distilled *in vacuo* with magnesium oxide to remove all the trimethylamine and ammonia present. The distillate contained 100 mg. of nitrogen almost entirely in the form of trimethylamine, identified by converting it

to its aurichloride which was analyzed. The residue in the distilling flask, after filtration and acidification with hydrochloric acid, was treated as described above, 3.07 gm. of the aurichloride of trimethylamine oxide being obtained. This amount corresponded to 80 per cent of the undetermined nitrogen, excluding that recovered as trimethylamine, in the distillate from the magnesium oxide. The aurichloride melted at 255° with decomposition. The melting point is given by previous workers as 250–257°. On analysis of the aurichloride, there was found, Au, 47.7 per cent; N, 3.39 per cent. Calculated for $(CH_3)_3NO \cdot HAuCl$, Au, 47.5 per cent, N, 3.37 per cent. The picrate prepared from a portion of the above aurichloride melted at 197°. Previous workers give it as 187–202°, depending upon the rapidity with which it is heated.

A further identification of the existence of trimethylamine oxide was made by heating the urine with sodium hydroxide and zinc dust and determining the trimethylamine liberated. In the case of each of the urines studied, an amount of base was liberated corresponding to the total undetermined nitrogen present.

Trimethylamine oxide, as a chemical entity, was first prepared and described by Dunstan and Goulding (7) in 1894. They formed it by the interaction of methyl iodide and hydroxylamine and later showed that it could be more easily prepared by treating trimethylamine with a 3 per cent aqueous solution of hydrogen peroxide and allowing the mixture to stand at room temperature for 24 hours. Other amine oxides were prepared in a similar manner. Since the initial work of Dunstan and Goulding, the amine oxides have been established as definite, stable chemical compounds by a number of workers (14). The structure of trimethylamine oxide has also been studied by several investigators, the last of whom, Noyes (21), considers it as $(CH_3)_3N::\overset{..}{O}$ with a double union between the nitrogen and oxygen atoms. Ionization studies by the same investigator indicated that the compound may or may not be combined with water when in solution.

Although the presence of trimethylamine oxide in urine has never been heretofore recorded, this substance has been found in other biological materials. Suwa (24) isolated a substance from

the muscles of the dogfish (*Acanthias vulgaris*) which he definitely showed to have the composition and properties of trimethylamine oxide. Henze (13) obtained the substance from the muscles of cephalopods. More recently Poller and Linneweh (22) have found it in the muscle and roe of the herring (*Clupea harengus*). One might expect, therefore, that trimethylamine oxide might be of quite common occurrence, and its appearance in the urine of *Lophius* is thus not surprising. Although no attempt has been made to isolate trimethylamine oxide from the muscle of *Lophius*, its occurrence in the sources cited above renders it very probable that it is likewise present in the muscle of *Lophius* and that this is the source of the material found in the urine. It is possible also that it may have, in part at least, an exogenous origin, being derived from the numerous fishes and Crustacea which *Lophius* ingests. Although not demonstrated to appear there, it is not unlikely that trimethylamine oxide is also a constituent of the urine of the dogfish and herring and most probably of other animal species.

In passing, it may also be noted that the hitherto unisolated substance termed *aminol*, which is found in herring brine and has a bactericidal action in the presence of lime (3), may be trimethylamine oxide.

As regards the physiological significance of the occurrence of trimethylamine oxide in the lower vertebrates, nothing definitely may be said. From the high concentration in which it occurs in the urine—representing about half of the total urinary nitrogen—and the relatively high concentration in which it has been found in the muscles, it is but natural to conclude that it forms one of the main end-products of protein metabolism. It is possible that it may arise from betaine which, according to the investigations of Kutscher and Ackermann (19), is of wide occurrence in the lower vertebrates. Betaine, in fact, when fed to rabbits was found to be converted to trimethylamine by Kohlrausch (17). The oxidation of the trimethylamine would then take place according to the schema proposed by Ackermann, Poller, and Linneweh (2). As a result of a study of the reactions of trimethylamine oxide, the latter investigators have suggested that it is a biological hydrogen acceptor in the sense of the word as used by Wieland (29).

Trimethylamine.

One of the samples of urine collected in the Atlantic (Atlantic I) was kept in a metallic container which became badly corroded and gave a copious deposit of iron oxide to the urine. The urine assumed an alkaline reaction although the freshly collected urine is always acid. On examination, several weeks later, it was found to contain an amount of trimethylamine corresponding to 80 mg. of nitrogen per 100 cc. of urine. After this was deducted from the total undetermined nitrogen, there remained unaccounted for only 7 per cent of the total nitrogen. The trimethylamine was identified by distillation *in vacuo* with magnesium oxide, according to the procedure of Takeda (25)[2] by collecting in acid, evaporating the distillate to dryness, extracting with alcohol, and preparing the gold ' salt of trimethylamine hydrochloride. The product melted with decomposition at 228°: Au found, 49.6 per cent; N found, 3.46 per cent. Calculated for $(CH_3)_3N \cdot HAuCl_4$, Au, 49.4 per cent; N, 3.51 per cent.

The presence of so great an amount of amine in the urine (Atlantic I), was quite unexpected, as previous experiments had indicated the presence of only very small amounts of this substance. The explanation of its occurrence in so high a concentration in this specimen is easily accounted for when one considers the relative ease with which trimethylamine oxide may be reduced to the amine. It is, therefore, logical to assume that the trimethylamine found in this particular specimen of urine resulted from decomposition in alkaline solution of the trimethylamine oxide originally present.

Determinations of the amount of trimethylamine present in the other urines were also made by the method of Takeda (25). Woods Hole urine, several weeks after its collection, contained 9 mg. of trimethylamine nitrogen per 100 cc. of urine: after 10 weeks it contained 23 mg. per 100 cc. Atlantic I, as already stated, contained practically all the undetermined nitrogen in the form of trimethylamine. Atlantic II contained 7 mg. of amine

[2] Experiments with trimethylamine oxide, prepared by the action of hydrogen peroxide on trimethylamine, showed that the former compound underwent no decomposition when subjected to the procedure of Takeda (25).

nitrogen per 100 cc. a week after its collection and 70 mg. 5 weeks later. Aquarium urine contained 6 mg. of trimethylamine nitrogen, a week after its collection. The trimethylamine present in the urine most probably was absent in the fresh specimens, but resulted from the slow decomposition of the oxide, as is indicated by the increase in its content with time. The chemical nature of the oxide is such as to lead one to expect this decomposition. It is also possible that other compounds such as *novaine*, which Kutscher (18) isolated from crab extracts and which give trimethylamine on heating with alkali, are also present in the urine although no attempt was made in this investigation to isolate these substances.

Since at least 80 per cent of the undetermined nitrogen of the urine of *Lophius* could always be recovered in the form of tri-

TABLE II.

Inorganic Constituents of Urine of Lophius piscatorius.

The results are expressed in terms of millimols per liter of urine.

	Atlantic II.	Aquarium.
Cl	56*	188
P	8	8
Ca	2	3
Mg	38	108

* Heparinized plasma obtained from the blood of the fish from which the urine was collected had a chloride content of 177 mM per liter.

methylamine plus trimethylamine oxide, we may conclude that the latter substance constitutes the greater part of the hitherto undetermined nitrogen. The large and increasing amounts of the trimethylamine found in the urine after some weeks, but not present in the fresh material, result, no doubt, from the reduction of the oxide.

Inorganic Constituents.

Although the inorganic constituents of the urine of *Lophius* have been determined by several previous workers, it was thought of interest to compare the results obtained on freshly caught fish with the observations made previously on specimens kept in the laboratory. The results of these analyses for Atlantic II urine and for Aquarium urine are given in Table II.

The chlorides were determined by the Volhard-Harvey method, total phosphates by the method of Fiske and Subbarow, calcium and magnesium by the methods of McCrudden (12).

The obvious and noteworthy point about the concentration of the inorganic constituents, as given in Table II, is the relatively low values of the chloride, calcium, and magnesium of the urine obtained from the fish caught in the ocean compared to the concentration of these substances in the Aquarium specimen and the results reported by other observers on urine obtained in the laboratory. The freezing point lowering of the Atlantic I urine was 0.68° as compared to 0.83° for the Aquarium specimen. Their densities were 1.017 and 1.016 respectively. The high degree of ionization of the trimethylamineoxyhydrochloride helps to compensate for the low salt content of the Atlantic urine in maintaining the relatively high freezing point lowering of this urine as compared to that of the Aquarium specimen (21). In both cases, however, the freezing point lowerings are less than that of the blood.

The author is indebted to Professor E. K. Marshall, Jr., who suggested this problem, for aid and advice throughout the course of this investigation.

SUMMARY.

A study was made of the urinary constituents of the goosefish, *Lophius piscatorius*. The chief nitrogenous constituents of this urine were found to be creatine, creatinine, and amino acids. Ammonia, urea, and uric acid were present only in small amounts. The remainder of the urinary nitrogen was found to be made up chiefly of trimethylamine oxide which was isolated and identified. Specimens of urine obtained from the fish freshly caught in the Atlantic were found to contain much more nitrogen and much less inorganic salts than the specimens reported previously by other observers.

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HEXOSEMONOPHOSPHATE (ROBISON).

By P. A. LEVENE AND ALBERT L. RAYMOND.[*]

(From the Laboratories of The Rockefeller Institute for Medical Research, New York.).

In a previous communication[1] the conclusion was reached that the glucoside of the hexosediphosphate of fermentation possessed the γ-lactal ring and that the ester was 1,6-fructosediphosphate. The method employed consisted in measuring the rate of hydrolysis of the glucoside with very dilute acid at 100°, as under these conditions γ-glucosides hydrolyze rapidly and normal forms do not. While our paper was in press, one by Morgan and Robison[2] appeared in which the same conclusion was reached from a different attack and this helps to validate the method as applied to the phosphate esters of the sugars.

The present paper deals with the application of the same method to the Robison monophosphate which is formed in small amount during enzymic fermentations. Little is known of the structure of this ester. Meyerhof[3] has shown it to be largely an aldose, and it is most probably a glucose derivative. As to the allocation of the phosphate, only position (6) may be excluded, and this because the osazones of the Robison ester and of the Neuberg ester (which is fructose-6-monophosphate[1]) are different.

The glucoside was prepared from a pure sample of the Robison ester and submitted to hydrolysis under the conditions previously described. The very rapid hydrolysis which was observed made it certain that the glucoside had the <1,4> lactal ring. Moreover, as the reduction after 10 minutes accounted for practically all of the glucoside which was used, it was certain that only the

[*] National Research Fellow in Chemistry.

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[2] Morgan, W. T. J., and Robison, R., *Biochem. J.*, **22**, 1270 (1928).

[3] Meyerhof, O., and Lohmann, K., *Naturwissenschaften*, **14**, 1277 (1926).

one ring form was present. If in the original ester there was a small proportion of any other ester, then it must have been lost in the process of isolation.

The γ form of the glucoside and the fact that the ester is a glucose derivative immediately locate the phosphate radical on carbon atom (5) for otherwise the stable $<1,5>$ lactal ring would have formed. The Robison ester therefore has the formula (I):

$$
\begin{array}{l}
\left. \begin{array}{l}
\!-\text{CHOH} \\
\;\;\;| \\
\;\;\;\text{CHOH} \\
\text{O}\;\;| \\
\;\;\;\text{CHOH} \\
\;\;\;| \\
\!-\text{CH}
\end{array} \right. \\
\;\;\;\;\;| \\
\;\;\;\;\;\text{CH-O-PO}_3\text{H}_2 \\
\;\;\;\;\;| \\
\;\;\;\;\;\text{CH}_2\text{OH}
\end{array}
$$

I.
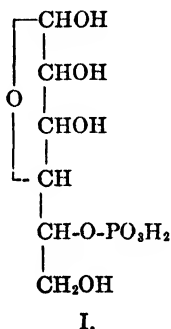
EXPERIMENTAL.

1. Robison Ester.—The preparation of the ester was described previously.[4] It gave an osazone decomposing at 137–138° and had the following rotations.

$$\text{Barium salt } [\alpha]_D^{25} = \frac{+\,1.13° \times 100}{1 \times 9.12} = +\,12.4°.$$

$$\text{Free acid } [\alpha]_D^{25} = \frac{+\,1.56° \times 100}{2 \times 3.04} = +\,25.7°.$$

Robison[5] gives $+12.5°$ and $+25.0°$ respectively. The Neuberg monophosphate is quite different, having rotations of $+0.4°$ and $+1.5°$ in the above cases.

The reduction as found by the Lehmann-Maquenne technique previously employed was only 66 per cent of that of an equimolal quantity of glucose which is a lower value than that given by Meyerhof.[3]

[4] Raymond, A. L., and Levene, P. A., *J. Biol. Chem.*, **79**, 628 (1928).

[5] Robison, R., *Biochem. J.*, **16**, 809 (1922).

2. Glucoside.—The preparation of the glucoside was similar to that of the diphosphate glucoside.   8 gm. of the dry barium salt of the monophosphate were added to 300 cc. of dry methyl alcohol containing 3.0 gm. of HCl gas (equivalent to 0.5 per cent free HCl).   The barium salt dissolved almost completely on shaking. A portion of the solution was rapidly filtered and used for the measurement of the optical rotation.   The solutions were kept at room temperature (about 25°) and the rotations measured in a 2 dm. tube are plotted in Fig. 1.   It is evident that the reaction was quite rapid, being 75 per cent complete in 2 hours and
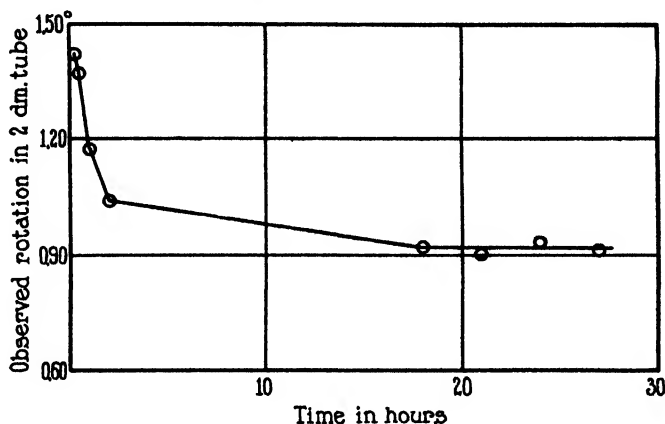
FIG. 1. Optical rotation during formation of methyl glucoside of Robison ester.

this at once suggests a <1,4> ring form.   There is no evidence of any secondary change, the slight variations being due to observational error.

For the isolation of the glucoside Morgan's[6] procedure was used, the hydrogen chloride being neutralized with a solution of barium hydroxide in methyl alcohol.   The precipitated barium salt was dried, dissolved in water, and precipitated with 4 volumes of absolute alcohol.   The solution and precipitation were repeated twice more and the product was then dried.   Yield 7.4 gm.   It

[6] Morgan, W. T. J., *Biochem. J.*, **21**, 675 (1927).

was perfectly white and gave very little reduction with Fehling's solution.

The chloride content of the crude salt was determined by analysis and a solution of the theoretical equivalent of silver sulfate was added. After removing the precipitate of silver chloride and

TABLE I.

Hydrolysis of the Methyl Glucoside of the Robison Monophosphate.

Time.	Reduction.	Increase.	Hydrolysis.
min.	*mg. glucose*	*mg. glucose*	*per cent*
0	0.50		
1	4.67	4.17	63
3	6.53	6.03	92
6	6 65	6 15	93
20	6.77	6.27	95
60	6.88	6.38	97
		6.59 Theoretical.	
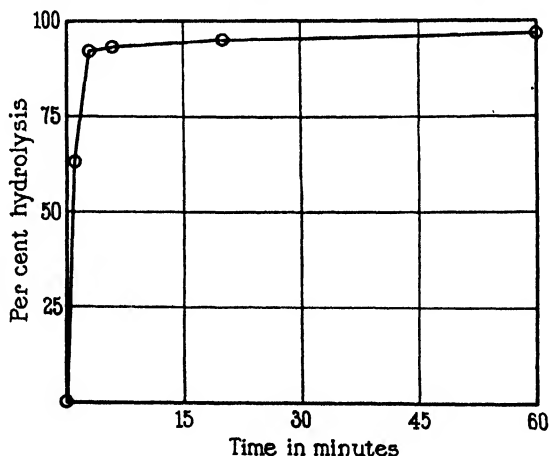
FIG. 2. Hydrolysis of glucoside of Robison ester with 0.1 N HCl at 100°.

barium sulfate by centrifuging, the solution was concentrated under reduced pressure to a small volume and alcohol was slowly added with stirring. The product was fairly soluble at an alcohol concentration of 35 per cent and almost completely precipitated at 80 per cent concentration.

Attempts at resolution into possible α and β forms were not successful with the small amount of material which was available. The analysis showed the product to be not completely methylated but the glucoside content was calculated from the methoxy figures.

0.1064 gm. substance: 0.0544 gm. BaSO$_4$.
3.290 mg.   "   : 17.260 mg. Mg$_2$P$_2$O$_7$.
0.0500 gm.   "   : 0.0268 gm. AgI.
     C$_7$H$_{15}$O$_9$PBa. Calculated. Ba   33.54, P 7.57, OCH$_3$ 7.57.
                       Found.   "   31.54, " 7.61, "   6.74.

Hydrolysis.—In performing the hydrolysis of the glucoside, no quantitative phosphate determinations were made as the phosphate cleavage was found qualitatively to be negligible. Hydrochloric acid, 0.1 N, and glucoside, 0.055 molal, were used, and the hydrolysis was done in a boiling water bath in sealed tubes as previously described for the diphosphate. The analytical technique was also the same.

In Table I are given the reduction data and the percentage hydrolysis as calculated from the reduction of the pure Robison ester. The percentage hydrolysis plotted against the time is also given in Fig. 2. It is evident from these that we are dealing with a γ-lactal ring, and, moreover, that this is the only ring form which is present.

SUMMARY.

On the basis of the rate of hydrolysis of the methyl glucoside of the Robison monophosphate it is concluded that the substance has the γ-lactal structure.

From this and the fact that the ester is an aldose derivative, it follows that the phosphoric acid radical is attached to carbon atom (5).

THE METABOLISM OF SULFUR.

XIV. A METABOLIC STUDY OF A CASE OF CYSTINURIA.

By HOWARD B. LEWIS AND S. ALLAN LOUGH.

(From the Laboratory of Physiological Chemistry, Medical School, University of Michigan, Ann Arbor.)

The subject of our study was a young man, American, 25 years of age, who first came under our observation in February, 1925. Cystinuria had been observed in 1922 by Dr. R. B. Gibson of the University of Iowa and the observation was confirmed in 1924 by Dr. W. C. Brasch of the Mayo Clinic, to whom we are indebted for the records of their investigations of this case. In April, 1927, cystine was still present in the urine in large amounts. On a number of occasions, the patient passed small cystine calculi. As far as known to us, no surgical procedure for the removal of calculi was ever necessary. We have not been able to obtain any record of familial cystinuria, although we have made no examination of the urines of the patient's family group. At the time of our observations, the subject weighed 61.3 kilos and was in good physical condition. For some months previous to our studies, 10 gm. of sodium bicarbonate had been administered daily. This was continued during the early part of our work but was later discontinued.

Determination of Cystine in Cystinuric Urine.

One of us has recently reviewed (1) the methods used for the determination of cystine in the urine and has reported a comparative study of the results obtained by the gravimetric method of Gaskell (2), the modification of Gaskell's method proposed by Magnus-Levy (3), and the colorimetric method of Looney (4). From aqueous solutions, cystine could be recovered by the gravimetric procedure satisfactorily (90 to 95 per cent). The precipitated cystine was shown to be of a high degree of purity, as demon-

strated by the close correspondence between the values obtained gravimetrically (Gaskell) and those obtained by the analysis of the precipitate either polarimetrically (Magnus-Levy), colorimetrically (Looney), or for sulfur content. In urines to which cystine had been added, a considerable amount of cystine (as determined colorimetrically) remained in solution after the precipitation with acetone (Gaskell) or alcohol (Magnus-Levy), and the precipitated cystine was not pure. When small amounts of cystine were added to normal urine, amounts comparable to those frequently present in the urine of cystinurics, the recovery was very unsatisfactory, the loss amounting to 40 to 50 per cent in some cases.

Tne solubility of cystine in urine, greater than that of cystine in water, long recognized, has been reinvestigated by Blix (5). He was able to demonstrate that two factors were important in increasing the solubility of cystine in urine, the presence of various inorganic salts and the protective action of certain urinary colloids, which prevented or delayed crystallization of cystine. Of the inorganic salts studied, the effect of calcium chloride was most marked; e.g., the solubility of cystine in 0.25, 0.50, and 1.0 N solutions of calcium chloride at 16–18° was 59, 97, and 151 per cent, respectively, greater than in water. In view of these findings, it is unfortunate that in the method of Gaskell and its modifications, calcium chloride is added to precipitate the phosphates prior to the alcohol or acetone precipitation. Thus in one application of Gaskell's method (6), 20 cc. of a 20 per cent solution of calcium chloride were added to 250 cc. of urine. With such an amount of calcium chloride, a considerable excess of calcium salts must have remained in the solution, which should have increased the solubility of cystine.

The colorimetric method of Looney (4), on the other hand, although it possesses the virtues of simplicity and speed, has been criticized on the basis of non-specificity. Okuda (7) has maintained that substances present in urine other than cystine may give an increased intensity of color with the uric acid reagent in the presence of sodium sulfite, and that Looney's method gives high results particularly with urines low in their content of uric acid. Andrews (8) noted that, under certain conditions, the colorimetric determination indicated the presence of more cystine

than was known to be present originally and concluded that the method could not be taken as an unequivocal measure of cystine content.

Since we have made use of the colorimetric method in our study, and since in the case of cystinuria here discussed the excretion of cystine was greater than in those cases reported previously (9), with the exception of the case of Magnus-Levy (3) during an acute infection, it seemed advisable to make a comparison of the various methods of cystine determination on a cystinuric urine in the same manner as previously reported when cystine had been added to a normal urine (1). The sample used gave the following figures on analysis, calculated on the basis of 100 cc. of urine: total nitrogen, 0.367 gm.; amino nitrogen, 0.020 gm.; total sulfur, 34.3 mg.; total sulfate sulfur, 17.3 mg.; organic ("neutral") sulfur, 17.0 mg. Direct colorimetric analysis by the method of Looney showed 47 mg. of cystine per 100 cc. The weights[1] of cystine obtained by Gaskell's method were 39, 37, and 37 mg. per 100 cc., respectively, in triplicate determinations. Analysis of the precipitated cystine showed the presence of 35, 33, and 34 mg. of cystine, respectively, by the polarimetric method; of 32, 31, and 33 mg. by the colorimetric method; and of 33, 36, and 34 mg. as calculated from the analyses for total sulfur content of the precipitate. The filtrates from the precipitation with acetone contained 16, 18, and 12 mg. of cystine as estimated colorimetrically. These results are in agreement with our earlier studies of the recovery of cystine added to normal urine in indicating that a considerable portion of the cystine (or substances reacting as cystine in the colorimetric determination) remains in solution in the filtrate in Gaskell's method. In the present case, the cystine sulfur as calculated from the direct estimation colorimetrically comprised about 73 per cent of the organic sulfur present, while the cystine sulfur as calculated from the gravimetric determination comprised only 51 per cent of the organic sulfur.

Although we realize the defects in the colorimetric method, we have estimated the cystine in this way and have also calculated the cystine from the "extra" organic sulfur on the assumption that the organic sulfur under normal conditions is relatively constant and independent of the diet. In certain cases, the cystine

[1] These analyses were made by Mr. R. H. Wilson of this laboratory.

sulfur as calculated from the cystine values obtained in the colorimetric determination *was in excess not only of the "extra" organic sulfur,* but also of the *total organic sulfur.* Looney also encountered similar difficulties since in the cystinuria studies of Looney, Berglund, and Graves (10), the cystine sulfur was 134 per cent of the neutral sulfur (p. 524, Table X), although recorded in the table as 100 per cent. Similar discrepancies occur elsewhere in their tables (p. 523, Table IX; p. 528, Table XII). In our own work, where such discrepancies are evident, we have preferred to rely upon the cystine as calculated from the "extra" organic sulfur, bearing in mind the criticisms already referred to (7, 8).

Other routine analytical procedures included the Kjeldahl method for total nitrogen, the Folin micro method for creatinine, gravimetric determination of the partition of sulfur, and Folin's colorimetric method for amino acids. The cystine and creatinine determinations were carried out on the same day the sample of urine was collected.

Origin of the Cystine Excreted in Cystinuria, Endogenous or Exogenous.

The source of the cystine excreted by the cystinuric, whether it be endogenous or exogenous in its origin, is not known with certainty. Although the quantity of protein in the diet definitely influences the cystine elimination (6, 11, 12), this exogenous factor is not the only factor involved, since cystine excretion continues on a nitrogen-free diet (11) or in fasting (10). The effect of protein ingestion is usually considered to be due to the presence of cystine in the protein and hence low protein diets are commonly advised, in addition to alkalinization of the urine through the use of sodium bicarbonate, in treatment to prevent recurrence of the formation of cystine calculi. So far as we have been able to discover, no diet, whose protein is of known cystine content, has been studied in its relation to the excretion of cystine in cystinuria, nor has there been made any comparison of the effects of diets of like protein (*i.e.,* nitrogen) content but of different cystine content. We have attempted to approach the question of the endogenous or exogenous origin of the cystine, which escapes the normal fate in metabolism in the cystinuric, by a study of this sort.

The diet chosen was a milk diet, supplemented by apples to increase the caloric value, to give bulk, and to insure an adequate indigestible residue to prevent constipation. The subject ingested 2 quarts of milk and 400 gm. of apples daily for a period of 6 days, on the last 5 of which urine samples were collected for analysis. As a control, we were fortunate in securing a normal individual of approximately the same age and weight (60.0 kilos) as the cystinuric subject, who ingested the same diet of milk and apples for a 5 day period. The results of these metabolic studies are presented in Tables I (normal control) and II (cystinuric).

The diet furnished approximately 9.87 gm. of nitrogen (milk, 9.68 gm.; apples, 0.19 gm.) and 0.645 gm. of sulfur (milk, 0.621 gm.; apples, 0.024 gm.). On the basis of the usual analyses, the milk ingested should have contained 62 gm. of protein, which would include, if the ratio of casein to lactalbumin in cow's milk is 5:1, 51.6 gm. of casein and 10.3 gm. of lactalbumin. Casein and lactalbumin contain 0.26 and 3.90 per cent of cystine respectively [13] according to analyses by the colorimetric method of Folin and Looney.[2] 2 quarts of milk should contain 0.525 gm. of cystine (0.134 gm. (casein) + 0.391 gm. (lactalbumin)), equivalent to 0.140 gm. of cystine sulfur. The total sulfur content of the apples is so low that these cannot be regarded as significant sources of cystine. The cystine content of the diet, as thus calculated, may be considered as maximal, since in many cases the ratio of casein to lactalbumin in cow's milk may be greater than 5:1 and the cystine figures are probably too high.[3]

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[2] Okuda [7] considers that the results obtained by the use of the method of Folin and Looney are too high. He found 0.32 per cent of cystine in casein by the Folin-Looney method and 0.17 per cent by his own iodometric method. In this laboratory, lactalbumin by the method of Folin and Looney has been found to contain 4.15 per cent of cystine (Wilson, unpublished data).

[3] Sherman and Woods [14], on the basis of biological analysis involving growth experiments with the white rat, believed that their results indicated the presence in casein of not less than 1.3 per cent nor more than 2.5 per cent of cystine or other sulfur-containing radicles, which are interchangeable with cystine in nutrition. However, in view of the failure to isolate cystine in significant quantities from casein [15] and of the low content of cystine in casein as shown by analyses by Van Slyke's partition method, Folin and Looney's colorimetric method, and Okuda's iodometric method,

As shown in Tables I and II, the average daily excretions of total nitrogen, creatinine, and total sulfur were very similar in the con-

TABLE I.

Subject C. Normal Control.

2 quarts of milk and 400 gm. of apples daily.

Date.	Volume.	N	Amino N.	Creatinine.	Total S.	Total sulfate S.	Organic S.
1925	*cc.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
May 5	1710	12.09	0.071	1.451	0.695	0.616	0.079
" 6	1790	11.21	0.070	1.500	0.696	0.618	0.078
" 7	1580	12.59	0.083	1.564	0.833	0.764	0.069
" 8	1270	11.98	0.070	1.468	0.764	0.703	0.061
" 9	1380	12.78	0 071	1.468	0.803	0.738	0.065
Average....		12.13	0.073	1.490	0.758	0.688	0.070

TABLE II.

Subject O. Cystinuric.

2 quarts of milk and 400 gm. of apples daily.　10 gm. of sodium bicarbonate divided into three doses daily.

Date.	Volume.	pH	N	Amino N.	Creatinine.	Total S.	Total sulfate S.	Organic S.	"Extra" organic S.	Cystine (Looney).	Cystine S.
1925	*cc.*		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Feb. 11	2900	7.6	11.08	0.544	1.450	0.806	0.398	0.408	0.338	1.011	0.270
" 12	2910	8.0	11.91	0.558	1.419	0.823	0.369	0.454	0.384	1.497	0.400
" 13	2370	7.4	11.27	0.487	1.402	0.729	0.367	0.362	0 292	1.319	0.352
" 14	1940	7.8	10.77	0.465	1.514	0.739	0.355	0.384	0.314	1.607	0.429
" 15	1940	7.4	10 28	0.306	1.481	0.657	0.319	0.338	0.268	1.462	0.390
Average.............			11.06	0.472	1.453	0.751	0.362	0.389	0.319	1.379	0.368

trol urine and in the cystinuric.　The ratios of total nitrogen to total sulfur were also very similar, 16.0 and 14.7 respectively.

we believe that further confirmation of these figures by chemical analyses or by isolation of sulfur-containing compounds capable of functioning in place of cystine in nutrition is necessary.　The possibility that methionine (16) is such a compound must be seriously considered, but it seems improbable that it can give rise to cystine in its metabolism.

Wolf and Österberg (17) in experiments with normal individuals on a diet whose protein was derived entirely from milk, but which contained slightly more nitrogen than the diet of our study, observed a urinary N:S ratio of 15.6. In our normal subject, however, the organic sulfur excretion was 0.070 gm. or 9.2 per cent of the total sulfur, as compared with an excretion of organic sulfur of 0.389 gm. or 48.3 per cent of the total sulfur by the cystinuric. If we assume the constancy of the organic sulfur under normal conditions and use as our basis of calculation the organic sulfur excreted by the control subject (since the two individuals were of approximately the same age and weight), the elimination of "extra" organic sulfur by the cystinuric was 0.319 gm. or 82 per cent of the total sulfur. The sulfur present as cystine, as calculated from the cystine determination by the colorimetric method, was 0.368 gm. or 94.6 per cent of the organic sulfur. This difference between the cystine sulfur as determined colorimetrically and the cystine sulfur calculated as "extra" organic sulfur may be due to the fact that the normal values for organic sulfur of the urine may include some cystine sulfur (10) or to the fact that colorimetric determination may give values for cystine which are too high. It should be noted that on the last 2 days of the experiment (Table II), the sulfur as calculated from the cystine determinations was *in excess of the total organic sulfur*. There is a fair correspondence between the values obtained directly and indirectly and there is no indication of any abnormality in the sulfur metabolism other than the excretion of cystine.

The relations between the cystine content of the diet and the cystine excreted in the urine are also of some interest. As detailed above, the diet was estimated to contain 0.525 gm. of cystine. The excretion of cystine was 1.379 gm. as directly determined or 1.194 gm. as calculated from the "extra" organic sulfur. It is evident that *the amount of cystine excreted was more than the amount of cystine present in the diet*. This indicates an *endogenous origin of a significant portion of the cystine excreted*. Both the normal and cystinuric subjects were in negative sulfur balance, the amount of urinary sulfur excreted being slightly in excess of the sulfur of the diet as calculated.

In the cystinuric, the amino acid nitrogen figures were very high, much higher than could be accounted for by the amino

nitrogen contained in the cystine excreted.   Thus the nitrogen content of the cystine was only 0.161 gm. or about 34 per cent of the total urinary amino nitrogen.   This suggests an abnormality of the metabolism of amino acids other than cystine.   It should be noted that there was a decline in the amino acid nitrogen as the experiment progressed.   These figures were higher than those observed in other periods (*cf*. Table III) and never

TABLE III.
Subject O.   Cystinuric.

Date.	Volume.	pH	N	Amino N.	Creatinine.	Total S.	Total sulfate S.	Organic S.	"Extra" organic S.	Cystine (Looney).	Cystine S.
A. Low protein mixed diet.											
1925	cc.		gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
May 5	1260	6.6	7.98	0 257	1.464	0.691	0.390	0.301	0.231	1.053	0 281
" 6	1400	6.4	8.04	0.316	1.520	0.673	0.373	0.300	0.230	0.978	0.261
" 7	2100	6.4	7.43	0.278	1.489	0.621	0.357	0.264	0.194	1.465	0.391
" 8	1460	6.4	6.75	0.252	1.572	0.666	0.402	0.264	0..194	1.592	0.425
" 9	1710	6.6	7.67	0.279	1.582	0.713	0 405	0.308	0.238	1.216	0.325
Average............			7.57	0.276	1.525	0.673	0.385	0.287	0.217	1.261	0.337
B. High protein mixed diet.											
May 19	2800	6.8	10.57	0.416	1.513	0.826	0.428	0.398	0.328	1.844	0.492
" 20	2380	6.8	10.26	0.374	1.485	0.761	0.433	0.328	0.258	1.726	0.460
" 21	2160	6.8	10.83	0.428	1.521	0.825	0.454	0.371	0.301	1.478	0.394
" 22	1740	6.6	11.28	0.359	1.547	0.801	0.490	0.311	0.241	1.342	0.358
" 23	2220	6.6	11.37	0.416	1.720	0.851	0.520	0.330	0.261	1.664	0.444
Average............			10.86	0.399	1.557	0.813	0.465	0.348	0.278	1.611	0.430

did we obtain values as great as during the first 2 days of the present study.   We have examined the urine of our subject repeatedly for the presence of tyrosine with uniformly negative results.   No attempt was made to detect diamines in the urine.

In a subsequent experimental study, the cystinuric ingested a uniform weighed mixed diet of lower protein content which was estimated to contain 45 to 50 gm. of protein, but which included

no milk.   This diet, while it contained less total protein than the milk diet already discussed, undoubtedly had a higher content of cystine since the protein was derived chiefly from wheat bread[4] and eggs.   Despite the higher cystine content of the diet, the eliminations of organic sulfur and "extra" organic sulfur (Table III, A) were appreciably less than in the experiment just discussed, 0.287 and 0.217 gm. as compared with 0.389 and 0.319 gm. The cystine as determined colorimetrically was also less, although it should be noted that on 3 of the 5 days, the cystine sulfur as calculated from the colorimetric determination was considerably in excess of the total organic sulfur.   It should also be noted that, despite the difference in the protein level, the inorganic sulfate elimination was essentially the same in both series of urines.

The subject later received a uniform weighed mixed diet of higher protein content, which was calculated to contain essentially the same amount of protein as the milk-apple diet previously eaten, but which was estimated to furnish a much greater amount of cystine, since its protein was derived from wheat products (crackers, bread, Shredded Wheat), eggs, meat (beef tongue), nuts (almonds and walnuts), and peas.   It was impossible to calculate exactly the cystine content of these foods, but on the basis of analyses of purified proteins derived from these sources, they must have supplied much more cystine than was furnished by the milk, with its chief protein, casein, of low cystine content.   Despite the higher content of cystine of this diet, the excretions of organic and "extra" organic sulfur (Table III, B) were essentially the same or slightly lower than when the milk-apple diet was eaten (Table II).   The similarity of the nitrogen excretions in the two experiments indicates that the nitrogen content of the two diets was essentially the same.   The cystine content of the urine as esti-

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[4] Jones, Gersdorff, and Moeller (13) reported 1.68 to 1.76 per cent of cystine in wheat gliadin.   Inasmuch as a sample of gliadin prepared by ourselves was found to contain 2.40 per cent, we secured other samples of gliadin from wheat through the courtesy of Dr. T. B. Osborne and Professor R. A. Gortner.   Both these samples gave results identical with our earlier value (Wilson and Lewis, unpublished).   Looney (18) subsequently repeated his earlier analysis and obtained 2.40 per cent as the cystine content of gliadin.   He also found a slightly higher content of cystine in wheat glutenin than did Jones and his coworkers.

mated colorimetrically was slightly greater on the mixed diet, but since the cystine sulfur as calculated from this determination was considerably in excess of the organic sulfur, we are inclined to rely more on the latter as an index of cystine excretion.[5]

A second control subject (77.0 kilos in weight) ingested the same diets as the cystinuric, *i.e.* the low protein mixed diet and the higher protein mixed diet, on the same days and complete urinary analyses were carried out. The results were typical of a normal individual in every respect. With average nitrogen excretions of 8.8 and 11.7 gm. daily, the average daily organic sulfur eliminations were 0.082 and 0.087 gm. respectively, thus demonstrating anew that in a normal individual this fraction of the urinary sulfur is independent of the diet, and justifying the use of a constant figure for the normal organic sulfur excretion in the calculation of the "extra" organic sulfur excretion by the cystinuric subject.

While we realize that each case of cystinuria undoubtedly presents individual peculiarities, we believe that the results of these studies on our patient indicate that the cystine excretion is largely endogenous in origin and is independent to a considerable extent of the cystine present in the protein element of the diet. It is well known that proteins exert a stimulating action on metabolism (specific dynamic action). Our results as presented in Table III show, as has been demonstrated by many others, an increase in organic sulfur (presumably cystine sulfur) which accompanies an increase in the level of protein metabolism. We would not be inclined to attribute this directly to the content of cystine of the diet, but rather to the effect of protein in stimulating some unknown endogenous metabolic process, whose abnormality in the cystinuric results in the excretion of a considerable portion of the cystine, which plays a rôle in body processes, unoxidized. Such a hypothesis would explain the fact that in most cases studied the cystinuric has been able to oxidize cystine ingested as such, while ingestion of high protein diets has increased the extent of

[5] It might appear that these high cystine values, as determined colorimetrically, might have been due to impurities in our reagents, which caused an increased color development. Analyses of a considerable number of normal urines, in which the same series of reagents was used, uniformly failed to demonstrate the presence of more than traces of cystine in these normal urines.

the cystinuria. It is our hope that further experimental work, designed to test the validity of this hypothesis, may be undertaken by others who have the opportunity to observe cystinuric subjects.

Our subject resembled most cystinurics previously studied in that he was able to oxidize almost completely cystine ingested as the amino acid itself. During a preliminary 4 day period, in which a diet of rather low protein content was eaten (average daily urinary nitrogen excretion, 8.0 gm.), the average daily urinary sulfur eliminations were 0.697, 0.392, and 0.304 gm. of total sulfur, total sulfate sulfur, and organic sulfur respectively. 2 gm. of cystine were then fed and for the next 24 hour period the excretions were 1.030, 0.681, and 0.349 gm. of total sulfur, total sulfate sulfur, and organic sulfur respectively. On the following day the sulfur excretion had returned to normal; i.e., 0.670, 0.356, and 0.314 gm. respectively. The cystine as determined colorimetrically for the fore period was 0.995 gm.; for the experimental cystine day, 0.881 gm.; and for the after day, 0.995 gm. The increase in the oxidized (sulfate) sulfur on the experimental day as compared with the fore period was 0.289 gm., equivalent to 54 per cent of the sulfur ingested as cystine. Similarly calculated, the rise in organic sulfur was 0.045 gm. or the equivalent of 8 per cent of the sulfur ingested as cystine. The remainder (36 per cent) of the sulfur fed as cystine was not recovered, probably because of failure of absorption or because of destruction by intestinal microorganisms. A second experiment, in which 3 gm. of cystine were fed, gave similar results.[6]

It has been observed by Looney and his associates (10) in the case studied by them, that sodium bicarbonate, in addition to increasing the solubility of the cystine in the urine and thus preventing the formation of calculi or cystine crystals, acted also in effecting a definite decrease in the total quantity of cystine excreted daily. As already stated, for some months previous to our observations the subject of our studies had ingested about 10 gm. of bicarbonate daily. For a period of 45 days, the bicarbonate

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[6] It should be noted that in these experiments the cystinuric subject was able to metabolize normally and almost completely 4 to 5 times the amount of cystine, which was estimated to be present in the milk-apple diet of Table I.

was discontinued. Although crystalline cystine was deposited from the urines in some cases during this period, no change in the daily excretion of cystine, which could be related to the withdrawal of the bicarbonate, was noted, nor did the excretion of cystine decrease when the administration of the bicarbonate was resumed.

SUMMARY.

1. Diets relatively high in protein, of approximately the same nitrogen content, but varying in their content of cystine, were ingested by a cystinuric patient. No greater excretion of cystine was observed after the ingestion of the diet containing the greater amount of cystine.

2. Comparison was also made of the effects of two diets, one of relatively high protein but of low cystine content, the second, of lower protein but furnishing a greater amount of cystine than the first. The excretion of cystine varied with the total nitrogen excretion and did not appear to be related to the cystine content of the diet.

3. The patient was able to oxidize completely 2 or 3 gm. of cystine ingested as the amino acid.

4. In view of these findings, we would suggest that the origin of the cystine excreted in cystinuria may be largely endogenous. Diets high in protein content are known to stimulate metabolism (specific dynamic action of protein). The effect of high protein diets in increasing the urinary output of cystine in cystinuria may be due similarly to a stimulation by the protein of some processes of endogenous metabolism, which results in the production of cystine, rather than to the failure to oxidize the exogenous cystine, present in greater amounts in a high protein diet.

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LIPID EXCRETION.

V. A STUDY OF THE PARTITION OF THE FECAL LIPIDS WITH SPECIAL REFERENCE TO BACTERIA.*

By WARREN M. SPERRY.

(From the Department of Biochemistry, The University of Rochester School of Medicine and Dentistry, Rochester, New York.)

It has been shown (1) that dogs, receiving an essentially lipid-free diet, excrete a small but definite amount of lipids in the feces. The bile has been ruled out beyond much question as the source of these fatty substances by the finding (2) that when it is diverted from the intestine there is marked increase rather than decrease in their amount. Another possible source is the microorganisms which make up a large part of the feces. The primary purpose of the investigation reported here was to determine how much of this lipid excretion is contained in the bodies of fecal bacteria. It was hoped that these bacterial lipids, if they were found to make up a large or significant portion of the excretion, might be uniform enough in percentage of total lipids and in composition to allow the determination of values which could be used with some degree of accuracy as corrective factors in the determination of lipid balances, particularly of cholesterol and the sterols. Channon (3) and the author (1) have pointed out that bacterial synthesis might account for the small negative cholesterol balances on the basis of which Gardner and Fox (4) and others have concluded that synthesis of cholesterol is possible in adults.

Two general methods of attack were available: (1) Bacter a, native to the intestine, might be grown in large amounts in pure cultures, their lipid content determined, and, from the amount of bacteria in the feces, the amount of bacterial fecal lipid computed;

* A preliminary report of this investigation was made before the American Society of Biological Chemists at Ann Arbor, April, 1928 (17).

or (2) the bacteria might be separated from the feces and their lipid content determined directly. The first of these procedures presented difficulties which seemed almost insurmountable. Practically no data are available for the amount of bacteria in dog feces, and even if there were, it is hardly likely that they could be applied to experiments with an unusual lipid-free diet, such as has been employed in this work. Certainly data obtained with humans could not be used, especially as there is a great variation in the results of different workers (see below). Since, as is well known, the intestinal flora may change to a considerable degree with changes in diet, it is conceivable that, in the event of such a change being rather slow, the situation in the 2nd week of an experiment might be quite different from that in the 1st. It seemed absolutely essential then that a determination of bacterial content be made of each sample of feces analyzed. But the only method available for doing this is actually to separate the bacteria, and once that is done one might as well determine their lipid content directly. Furthermore, to employ the first method at all accurately it would be necessary to know the distribution of various types of bacteria in the feces and the lipid content of each type. Such determinations would be very difficult and at best could be little more than approximate. At any rate, there would still be no assurance that bacteria, grown on synthetic media, would have the same lipid content as those of the same type grown in the intestine, since the lipid content of bacteria is known to vary considerably with the medium on which they are grown.

The second method overcomes all of these difficulties at once by determining the lipid content of the bacteria as they exist in the feces regardless of amount, type, or medium. It does involve the difficult separation of the bacteria, which, however, as pointed out above, is essential to the first procedure also. The only method available for this separation is that of fractional centrifugation first described by Strasburger (5) in 1902, and modified slightly by several investigators in the 12 year period following its introduction. Since this procedure is the basis of the work to be described, it is necessary to consider it in some detail. The method as described by Strasburger consisted in emulsifying thoroughly a small sample of feces with 0.5 per cent HCl (used to dissolve salts, soaps, *etc.*), centrifuging, stirring up the precipitated

solids with more HCl, centrifuging again, and repeating until the solids were reasonably free from bacteria as determined by the clarity of the final supernatant liquid and frequent microscopical examinations. Usually four such washings were required. The combined suspension was then centrifuged again at higher speed to remove any non-bacterial particles which might have escaped the first centrifuging, but this residue was not washed. Alcohol was added to the suspension and it was heated to coagulate the bacteria, which were centrifuged, washed·with ether, dried, and weighed.

The Strasburger method, either unchanged or with minor modifications, has been applied to the determination of bacteria in the feces of normal humans by Schittenhelm and Tollens (6), Lissauer (7), Steele (8), Friedenwald and Leitz (9), Sato (10), and Berger and Tsuchiya (11). All of these workers except Sato and Berger and Tsuchiya omitted centrifuging of the combined suspension; *i.e.*, they used only one serial fractionation. Steele and Friedenwald and Leitz filtered the suspension through cheese-cloth. Sato followed Strasburger exactly, while Berger and Tsuchiya went further and washed the second residue (obtained by centrifuging the combined washings of the first) once with another portion of 0.5 per cent HCl; *i.e.*, they employed a second short serial fractionation. MacNeal, Latzer, and Kerr (12) have used the method in a very extensive series of determinations with the important modification of carrying out three and frequently four serial fractionations with several washings of the residue obtained in each fractionation. This modified method has been employed with somewhat simpler technique by Mattill and Hawk (13) and Blatherwick and Hawk (14). Osborne and Mendel (15) have studied the fecal bacteria in rats by extracting undigested food-stuffs with a number of solvents and weighing the residue, which they believe is composed largely of bacteria.

Unfortunately there is a wide variation in the results obtained by these various investigators with feces of healthy humans on normal diets, varying in individual experiments all the way from 2.53 (Lissauer) to 45.5 (Strasburger), and in average values from 8.67 (Lissauer) to 34.03 (Blatherwick and Hawk), expressed as percentage dried bacteria in dried feces. As has been pointed out by Ehrenpfordt (16), these variations are probably due in large

part to the use of different centrifuging conditions. Most of the investigators give only approximate time and speed of centrifuging if they mention the point at all. Ehrenpfordt subjected duplicate samples of feces to the Strasburger procedure with two serial fractionations, in one case centrifuging each washing for 2 minutes at 1500 R.P.M., and in the other, 10 minutes at the same speed. He obtained with the shorter time an average of 25.31, and with the longer 14.75 per cent. It is obvious that for exact repetition it would be necessary to duplicate not only total time and maximum speed, but also time of acceleration and retardation, size of cups, and distance from point of suspension to center of centrifuge head. Since such exact duplication in different laboratories would be well nigh impossible, it would seem almost essential to control stages of the separation by microscopical examination. Such control has been found necessary in this investigation because of variations in different samples of feces. It has not been found possible to adopt a definite procedure and use it without microscopical control. Most of the investigators using the Strasburger method mention some use of the microscope in confirming the completeness of separation but the impression is obtained in most cases that the microscope is used only occasionally and for the most part only in examining the residue for freedom from bacteria —not in examining the bacterial suspension. Thus MacNeal, Latzer, and Kerr (12), who have done the most extensive work, say in a note, "In case of uncertainty [concerning freedom of residue from bacteria] microscopic examination should be resorted to. This is especially necessary before one has become familiar with the method." Ehrenpfordt in his study of effect of time of centrifuging, examined residues and bacteria (after precipitation by alcohol) and found with the short centrifuging period many non-bacterial particles in the bacterial fraction and with the long centrifuging period a great many bacteria in the residue. It is difficult to understand how many of the authors quoted above, using one or two serial fractionations with no microscopical control, can have obtained such complete separations as they claim, especially in view of the fact that the results vary so widely and the finding in this work that ordinarily ten to fifteen serial fractionations, involving twenty-five to forty-five settling (see below) periods, have been necessary to achieve anything like a complete separation.

EXPERIMENTAL.

The general plan was the same as that described in previous papers (1, 2). Dogs, used exclusively as subjects, were fasted 2 days, placed on the experimental, lipid-free diet for 2 or 3 more, or until they were eating well, and then fed charcoal to mark the beginning of the experiment, which was continued as long as the animals were eating reasonably well, not losing much weight, and were otherwise apparently healthy. In some cases a tendency toward diarrhea was evidenced. Usually it lasted but a day or two and the experimental feeding was continued; in a few cases it necessitated termination of the experiment. For the most part the stools were firm. In general the dogs did not evince as good an appetite as in previous work, only two taking the diet well for 4 weeks. However, it has not been possible to demonstrate any difference in lipid excretion between the 1st and 4th or 5th weeks of lipid-free diet feeding; so the 1st week is probably as valuable an experiment as any. With the long fore period, lasting nearly a week, the effect of previous feeding must be very small.

The diet was the same as described before (2), consisting of extracted casein, sugar, Vitavose,[1] bone ash, and salt mixture. Liebig's meat extract was fed in some cases. The casein was analyzed for total lipid content as described before. Different lots used in this work contained 3, 1.5, and 4.5 parts per 10,000 respectively of petroleum ether-soluble material, amounts too small to have any influence on the results obtained. In one case the casein was not analyzed but the mixed diet containing it was found to contain 2 parts per 10,000. Since this was a bit higher than expected on the basis of the casein and Vitavose content, samples of sugar were subjected to the same treatment (digestion with stong alkali, acidification, extraction with ether, and extraction of the ether extract with petroleum ether). A petroleum ether extract equivalent to 2.3 parts per 10,000 was obtained or 1.5 parts per 10,000 of the total mixed diet. Apparently a small amount of petroleum ether-soluble, non-lipid material is formed by the action of alkali on sugar. It may be that the small amounts of petroleum ether-soluble material usually found on analysis of

[1] The Vitavose used in these experiments was furnished through the courtesy of E. R. Squibb and Sons.

casein, even after long periods of extraction with alcohol, are formed in a similar fashion. At any rate it is evident that the amounts of lipid present in the diet are far too small to affect the results.

The method of collecting, separating, and analyzing the feces has been described previously (17) and need not be described in detail here. In the first three experiments carried out 0.55 per cent HCl was used as the suspending solution. These experiments are not included in the averages because of the probability that the relatively high acidity caused some breakdown of bacteria.[2] Mercuric chloride was not added to the solution under which the feces were collected in the next eight experiments. The note of Jordan (18), stating that a large increase in the bacteria of feces may occur during standing after excretion, casts considerable doubt on the results of these experiments and they also have been omitted from the averages. The hair fraction was not analyzed in the last nine experiments because it was found consistently to contain a negligible quantity of lipids.

It has been found since publication of the method that in some experiments three or even four washings are required in the first and second serial fractionations. Also more serial fractionations and of course more individual settling periods have been found necessary in several experiments. In one, eighteen serial fractionations comprising 59 individual settling periods were required for a satisfactory separation, but usually ten to fifteen serial fractionations are sufficient. As indicated in the preliminary paper, the final combined non-bacterial and bacterial fractions of all experiments were examined carefully under the microscope. In one experiment a number of large crystals was seen in smears of the packed and washed bacterial fraction. These could not have been present before concentration and they were identified as calcium phosphate. With this exception the separation in all

[2] The author is indebted to Dr. Ralph R. Mellon of the Institute of Pathology, Western Pennsylvania Hospital, Pittsburgh, for pointing out the danger in the use of HCl and for suggesting the use of formaldehyde to coagulate the bacteria. Formalin, enough to make the bacterial suspension 4 per cent formaldehyde, was added in four of the experiments but it was not found essential and was abandoned in the remaining experiments.

experiments was estimated to be at least 90 per cent complete. Complete separation of course is impossible by any sedimentation method because there are some large bacteria which settle more rapidly than many non-bacterial particles and some non-bacterial particles which settle more slowly than a large proportion of the bacteria. A great many particles of varying size but fairly uniform spherical shape were seen in most of the preparations. Many of these had the appearance of budding yeast cells but Dr. Bayne-Jones and other members of the Department of Bacteriology of this school were unable to identify them with any known micro-organisms. These particles disappeared almost instantly in the presence of acid. With the exception of a few of smallest size they seemed heavy and were easily separated from bacteria and included in the residue. Dr. Bayne-Jones has examined a number of typical bacterial and non-bacterial fractions and confirms the author in the view that the separations are probably over 90 per cent complete. It seems reasonable to assert that the separations must be at the very least as good as those upon which our knowledge of the bacterial content of feces is based, since many more serial fractionations were employed in these experiments than in any reported previously and all stages were controlled carefully with the microscope

Three principal fractions are obtained: bacterial, non-bacterial solids, and supernatant solution from the centrifuging of the bacteria. To the last are added ether washings of all vessels used in the separation and hence it must contain any lipids in free suspension or solution. It will be referred to as the soluble fraction hereafter. In seven of the experiments these three fractions (together with the hair and petroleum ether extract of dried bacteria where they were determined) were digested separately with strong alkali, the lipids were extracted, separated into unsaponifiable and fatty acid fractions, and the fatty acid fractions into solid and liquid fractions as described previously (1, 2).

One of the most interesting results of the work, as discussed below, is the finding that a relatively small portion of the lipid excretion is present as free lipids in suspended or soluble form. Most of these excreted fatty substances are present in such structures that they can be removed from an aqueous suspension by settling or centrifuging. There are three possible forms in which

the lipids may be present: (1) as true constituents of cells (bacterial, desquamated epithelial, *etc.*), (2) adsorbed on cells or other particles, or (3) as alkaline earth soaps (in the non-bacterial fraction).   In an attempt to obtain information on this point a different method of lipid analysis was employed in the last nine experiments.   The bacterial and non-bacterial suspensions were evaporated, if necessary,[3] at room temperature to about 500 cc. with a fan, acidified to Congo red with N H_2SO_4, and extracted with ether in a continuous extractor of the conventional type.   A narrow extraction cylinder, 4.5 cm. in diameter and 48 cm. high, was employed to insure thorough contact between ether and suspension.   Moreover, ether entered through a small tube with an opening at the bottom of the cylinder and the mixture was agitated rather rapidly by a power stirrer.   Under these conditions the mixing of ether and suspension was excellent.   It was found by trial that when the ether no longer picked up color in circulating the extraction was complete; but in each case it was carried on several hours longer, usually 12 to 15 hours in all.

The ether solutions were taken to dryness and the residues extracted overnight with petroleum ether to obtain the extractable lipids, which were weighed and separated into neutral and fatty acid portions.   The neutral portions were saponified and again separated.   The extracted suspensions were digested with strong alkali and the lipids were separated and analyzed as was done with the unextracted suspensions in previous experiments.   (See the diagram below.)

DISCUSSION.

In Table I are given average, low, and high values for all of the data except those obtained in the study of the extractability of the bacterial and non-bacterial lipids.   Lack of space does not permit a detailed presentation.   In all, twenty-seven experiments have been performed.   Of these three are omitted from the averages because the HCl used as suspending medium may have broken down a portion of the bacterial cells; eight are omitted because no antiseptic was employed to prevent the growth of bacteria in the

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[3] It is difficult to remove the packed bacteria from the centrifuge bottles, a liter or more of wash water sometimes being required.

feces after excretion. When the various percentage values from these eight experiments are averaged in with those from the sixteen experiments reported in Table I, the resulting averages are almost identical with the averages from the sixteen alone. Regardless of the possibility of error in this group of eight experiments, no appreciable difference in the results from them and from the remaining better controlled experiments can be observed, from which one may be justified in the conclusion that actually no error of the sort in question occurred. Though these experiments in which no antiseptic was added are of no value by themselves, taken in conjunction with the others, with which they agree and in which growth of bacteria was prevented, they acquire a certain amount of value, and it seems legitimate to consider the results given in Table I to be worth somewhat more than they would be as averages of sixteen experiments alone.

Total Lipids.—This value represents the sum of the various lipid fractions. The results are slightly lower than obtained in previous work (2), but the dogs used in this investigation were much smaller. Where their average weight was 4.6 kilos, in preceding experiments the dogs used averaged between 8 and 9 kilos. It might be expected that there would be some relationship between body weight and total excretion, although no such relationship could be found in previous experiments. It should be noted that the decrease in total lipids is far smaller than would be expected if the excretion were directly proportional to the body weight.

Bacterial Lipids.—It was primarily to determine the proportional amount of bacterial lipids in the lipid excretion that this work was undertaken. A rather wide variation in the percentage of bacterial lipids in total lipids is evident. The data have been subjected to a statistical analysis according to the method of Scott (19). The results are plotted according to his scheme in Chart I. They have been divided into groups of four for analysis, in the order in which they were obtained. Individual experiments are plotted with open circles, the means of successive groups with dots, the mean deviations of the means of these groups with vertical open bars, the mean deviation of the mean of the cumulative series as a solid black field, the cumulative mean with the horizontal open bars, and the final mean of the whole series with a dotted line.

TABLE I.

Partition of Fecal Lipids.

No. of experiments.		Mean.	Low.	High.
16	Weight of dogs, *kg.*	4.6	2.6	6.6
15*	Total lipids, *gm.*	1.537	0.769	2.622
15*	Bacterial lipids, *gm.*	0.621	0.365	1.179
16	" " in total lipids, *per cent*	40.4	27.7	54.8
15*	Non-bacterial lipids, *gm.*	0.829	0.356	1.420
16	" " in total lipids, *per cent*	53.4	32.3	67.3
23*†	Soluble and suspended lipids, *gm.*	0.062	0.019	0.283
24†	" " " " in total lipids, *per cent*	5.0	0.9	17.0
14‡	Petroleum ether extract of bacteria, *gm.*	0.036	0.006	0.168
14‡	" " " " " in total lipids, *per cent*	2.4	0.5	7.1
17§	Hair " *gm.*	0.022	0.004	0.052
17§	" " in total lipids, *per cent*	1.8	0.3	5.0
15*	Total unsaponifiable material, *gm.*	0.463	0.153	0.746
16	" " " in total lipids, *per cent*	29.7	19.9	38.2
15*	Bacterial unsaponifiable material, *gm.*	0.190	0.060	0.332
16	" " " in total unsaponifiable material, *per cent*	40.3	24.7	64.8
16	Bacterial unsaponifiable material in bacterial lipids, *per cent*	29.9	16.5	47.6
15*	Non-bacterial unsaponifiable material, *gm.*	0.254	0.093	0.441
16	" " " in total unsaponifiable material, *per cent*	55.5	35.1	75.3
16	Non-bacterial unsaponifiable material in non-bacterial lipids, *per cent*	30.7	23.0	42.9
11*‖	Total fatty acids, *gm.*	0.874	0.508	1.263
12‖	" " " in total lipids, *per cent*	62.2	55.3	70.3
11*‖	Bacterial fatty acids, *gm.*	0.390	0.264	0.607
12‖*	" " " in total fatty acids, *per cent*	45.1	33.8	61.6
12‖	Bacterial fatty acids in bacterial lipids, *per cent*	67.7	57.8	75.8
11*‖	Non-bacterial fatty acids, *gm.*	0.456	0.244	0.780
12‖	" " " in total fatty acids, *per cent*	52.2	31.9	66.1
12‖	Non-bacterial fatty acids in non-bacterial lipids, *per cent*	63.4	52.9	71.0

TABLE I—*Concluded.*

No. of experiments.		Mean.	Low.	High.
7	Ratio, liquid to solid fatty acids in bacterial fatty acids.	1.772	1.171	2.710
7	Ratio, liquid to solid fatty acids in non-bacterial fatty acids.	1.612	0.985	2.560

* In one experiment about half the feces was lost. The weights of fractions from this experiment are omitted from the averages but the percentages are included.

† Values for soluble and suspended lipids from the eight experiments where $HgCl_2$ was not added are included.

‡ Petroleum ether extract was not determined in the last nine experiments and the experiment referred to in the asterisk (*) note above. Values from the eight experiments where $HgCl_2$ was not added are included.

§ Values from all experiments except the last nine and the one referred to in the asterisk note above are included.

‖ In four experiments there was a considerable loss of fatty acids in course of the study of extractability. These experiments are omitted.

The purpose of such analysis is to ascertain whether enough experiments have been done to determine a mean which would not be changed by more experiments. As expected from the variations, such a point has not been reached in this work as shown by the small change in the cumulative mean caused by reckoning in the mean of the last group, but the relative stability of the cumulative mean makes it fairly certain that a point has been reached from which no great variation would occur no matter how many experiments were carried out. Though probably not enough experiments have been carried out to establish the *exact* true mean, it seems fairly certain that this value does not lie far from 40 per cent. It is obvious, however, that the variations are too great to allow the use even of the exact mean as a corrective factor in individual lipid balance experiments, as had been hoped would be possible.

Non-Bacterial Lipids.—In most experiments the bacterial and non-bacterial fractions make up 90 per cent or more of the total lipids; hence the variations of the percentage of non-bacterial lipids in total lipids are about the same as for the bacterial lipids and statistical analysis would lead to the same sort of conclusion.

About 53 to 54 per cent of the total excretion on the average is contained in non-bacterial solids.

Soluble and Suspended Lipids.—Perhaps the most outstanding result of the work is the finding in general of very low values for this fraction.   All experiments, except those in which HCl was used, are included in the average, since the non-addition of $HgCl_2$ could hardly have had any effect on the soluble or suspended lipids. Since ether washings of all vessels used in the separation were added to this soluble fraction, it seems certain that at most but a small portion of the fecal lipids is present in suspension or solu-
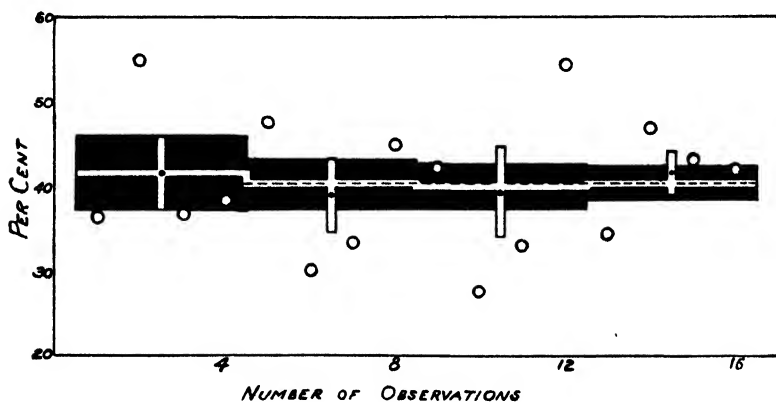
CHART I.   Open circles represent individual experiments; the dots, means of successive groups; vertical open bars, the mean deviations of the means of these groups.   The mean deviation of the mean of the cumulative series is shown by the solid black field, the cumulative mean by horizontal open bars, and the final mean of the whole series by the dotted line.

tion.   Moreover, these results are the best possible proof that no appreciable lysis of bacterial cells occurred during the long and rather strenuous treatment to which they were subjected.

Petroleum Ether Extract of Bacteria.—The failure to find any appreciable amount of lipids in suspension raised the question whether they might be carried down mechanically, adsorbed to bacteria or other particles.   In most of the experiments smears of packed bacteria after washing and centrifuging were stained with Sudan IV and examined. . No lipid particles could be made out. As a further check on the possibility of loose adsorption of lipids by

bacteria, the dried bacterial fractions in fourteen experiments were washed with three portions of petroleum ether for about 7 minutes. In all cases the amounts extracted were relatively very small, averaging only 2.4 per cent of the total lipids. If any conclusions are justified by such an experiment it would seem that the bacterial lipids are not easily extracted by petroleum ether and that loose adsorption is not indicated. On the other hand it may be that the petroleum ether simply did not penetrate the masses of dried bacteria. Since the bacteria were spread out in a rather thin layer on the surface of a large flask, however, there must have been a good deal of contact and one would expect more lipids to have dissolved than did, if they were in a state to be reached easily by the petroleum ether.

Hair.—A surprisingly uniform amount of hair was found in the feces, averaging about 1.5 gm. (dried) per week. This was analyzed as a separate fraction in seventeen of the experiments and was found to contain a negligible amount of lipids.

Unsaponifiable Material in Total Lipids.—The percentage of unsaponifiable material in total lipids is in agreement with previous work. The average of 29.7 per cent compares favorably with the values 33.1, 30.9, and 31.4 which were found in Series 3, Series 6, and bile fistula experiments respectively (2). There is somewhat more variation in the results than in the last mentioned experiments, perhaps to be expected when it is considered that there may be a summation of errors here since the total unsaponifiable value is obtained by adding the various unsaponifiable fractions.

Unsaponifiable Material in Bacterial Lipids.—In general the percentage of bacterial unsaponifiable material in total unsaponifiable material is in close agreement with the percentage of bacterial lipids in total lipids, both in individual experiments and averages, indicating that the percentage of unsaponifiable material in the bacterial lipids must be fairly uniform. Such is found to be the case. Rather wide extremes are observed, it is true, but ten out of sixteen of the experiments give percentages lying within 6 of the mean.

Unsaponifiable Material in Non-Bacterial Lipids.—The situation here is the same as in the case of the bacterial lipids. The percentage of non-bacterial unsaponifiable material in total unsaponifiable material is in close agreement with the percentage of

non-bacterial lipids in total lipids in individual experiments and averages, and as expected the percentage of non-bacterial unsaponifiable material in non-bacterial lipids is relatively uniform. There is slightly less variation in these results than in the corresponding ones for the bacterial fractions, eleven out of sixteen lying within 6 of the average.

From these data the conclusion seems justified that there is no essential difference in distribution of unsaponifiable material between the bacterial and non-bacterial portions of the fecal lipids.

Fatty Acids in Total Lipids.—The results are fairly uniform and in agreement with previous work.   The average percentage of fatty acids in total lipids confirms the results of Series 3, Series 6, and 1 ile fistula experiments (2) where the averages were 61.9, 59.5, and 63.1 respectively.   A loss of about 10 per cent of the total lipids on the average occurs during separation of unsaponifiable material and fatty acids.

Fatty Acids in Bacterial and Non-Bacterial Lipids.—As with the unsaponifiable fraction there is a rather striking agreement for the most part between the percentages of both bacterial and non-bacterial fatty acids in total fatty acids and the corresponding percentages of bacterial and non-bacterial lipids in total lipids. As expected from these results, the percentages of bacterial and non-bacterial fatty acids in the bacterial and non-bacterial lipid fractions are for the most part quite uniform, averaging 67.7 for the bacterial and 63.4 for the non-bacterial fraction.   These values are slightly higher than the average percentage of total fatty acids in total lipids.   The difference is due in the main to the inclusion in total lipids of values for the soluble, petroleum ether extract, and hair fractions, the fatty acid content of which was not included in the corresponding total fatty acid fractions.

Fatty Acid Composition.—In seven of the experiments the fatty acid fractions were separated into solid and liquid components by the modified Twitchell method described previously (1). The results as expressed by ratios of liquid to solid acids vary rather widely, due probably to errors involved in separating relatively small amounts; but the averages are in close agreement with each other and with the values 1.65 and 1.43 found in Series 3 and Series 6 respectively (2).

In summary it may be stated that the foregoing study of the

Fecal suspension ——— Soluble fraction

Bacterial fraction
Extracted with ether before saponification

Non-bacterial fraction
Extracted with ether before saponification

Suspension
Digested with strong NaOH

Unextracted lipids
Separated

Unsaponifiable fraction

Fatty acids

Extracted lipids
Separated

Free fatty acids

Neutral fraction
Saponified and separated

Unsaponifiable fraction

Fatty acids

Extracted lipids
Separated

Free fatty acids

Neutral fraction
Saponified and separated

Unsaponifiable fraction

Fatty acids

Suspension
Digested with strong NaOH

Unextracted lipids
Separated

Unsaponifiable fraction

Fatty acids

TABLE II.

Extractability of Lipids.

No. of experiments.		Mean.	Low.	High.
		per cent	*per cent*	*per cent*
8*	Bacterial lipids extracted.	49.9	21.0	83.5
9	Non-bacterial lipids extracted.	83.9	51.0	96.8
7*†	Free fatty acids in extracted bacterial lipids.	31.9	6.0	58.4
9	Free fatty acids in extracted non-bacterial lipids.	29.8	8.4	55.8
7*‡	Unsaponifiable material in neutral extracted bacterial lipids.	66.6	30.1	80.8
8‡	Unsaponifiable material in neutral extracted non-bacterial lipids.	54.7	38.0	72.3
7*‡	Unsaponifiable material in total extracted bacterial lipids.	40.3	28.3	49.8
8‡	Unsaponifiable material in total extracted non-bacterial lipids.	33.2	22.9	46.4
4*§	Fatty acids in neutral extracted bacterial lipids.	27.3	23.9	29.5
5§	Fatty acids in neutral extracted non-bacterial lipids.	38.0	21.4	53.0
4*§	Fatty acids in total extracted bacterial lipids.	57.9	49.3	69.0
5§	Fatty acids in total extracted non-bacterial lipids.	60.6	46.6	70.4
7*‡	Unsaponifiable material in unextracted bacterial lipids.	29.8	28.0	34.8
7†‡	Unsaponifiable material in unextracted non-bacterial lipids.	32.6	23.3	41.0
7*‡	Fatty acids in unextracted bacterial lipids.	65.0	54.9	70.5
7†‡	Fatty acids in unextracted non-bacterial lipids.	59.8	54.8	72.1

* In one experiment the bacterial fraction was not extracted with ether before saponification.

† Unextracted non-bacterial lipids were added to bacterial extracted neutral fraction by mistake in one experiment.

‡ Unextracted bacterial and non-bacterial lipids were added to extracted bacterial and non-bacterial neutral fractions after saponification in one experiment.

§ In four experiments there was a considerable loss of fatty acids during distillation, which was carried out to remove volatile acids. The volatile acids removed were negligible in quantity and the loss was due apparently to a failure to reextract all the fatty acids after the distillation. These experiments are omitted.

partition of unsaponifiable material and fatty acids in bacterial and non-bacterial fecal lipids demonstrates no appreciable difference in the two fractions. Such a finding would seem to indicate that the fecal lipid excretion represents some sort of secretion into the intestine where it is adsorbed both by bacteria and non-bacterial particles. On the other hand the proportions found are not unusual in living cells generally and it may well be that the striking similarity in the composition of the two fractions is simply a coincidence. It seemed possible that a study of the extractability of the lipids in the two fractions might help determine whether this lipid excretion represents an adsorbed secretion of suspended lipids or lipids present as constituents of cellular structures. In the last nine experiments a special procedure was employed as described under "Experimental." The accompanying diagram represents the steps of the separation schematically and explains the origin of the various fractions listed in Table II.

Extractability of Lipids.—The results show an unmistakable greater ease of extraction of non-bacterial than of bacterial lipids. At its face value this situation would seem to indicate that the bacterial lipids are more firmly held than the non-bacterial and might be taken as evidence that the bacterial lipids are to a large extent constituents of the cells, while the non-bacterial lipids are to a considerable extent adsorbed. How far such a line of reasoning is justified is a question. It is conceivable though not probable that adsorbed lipids might be more firmly held than true constituents of cells. Moreover, if the surface membranes of cells are largely lipid in nature, one might expect them to be readily removed by a powerful lipid solvent such as ether. A little evidence that something of this sort occurs in the case of the bacterial fraction is presented below.

Free Fatty Acids.—One of the purposes of these extraction experiments was to determine whether an appreciable portion of the non-bacterial lipids was made up of alkaline earth soaps. The results show that on the average 29.8 per cent of the extractable lipids is made up of free fatty acids which might have been present as soaps. The possibility that this moiety represents soaps cannot be eliminated, but the fact that in the bacterial fraction, where no calcium soaps could be present except by adsorption, the corresponding percentages average 31.9, makes it rather more

logical to assume a certain amount of hydrolysis occurring during the long separation period.

Unsaponifiable Material and Fatty Acid Partition in the Extracted and Unextracted Lipids.—The results are all in accord with the hypothesis that the portion of lipids extracted from the bacterial fraction is richer in unsaponifiable material and poorer in fatty acids than the unextracted portion, while in the case of the non-bacterial fraction the proportion of unsaponifiable and fatty acids is almost identical in the extracted and unextracted portions. From Table II it is evident that the percentages of unsaponifiable material in the extracted bacterial lipids are higher than the corresponding percentages in the extracted non-bacterial lipids or the unextracted bacterial lipids, while the percentages of fatty acids in the extracted bacterial lipids are lower than the corresponding percentages of fatty acids in the extracted non-bacterial lipids and the unextracted bacterial lipids.   Moreover, the percentage of unsaponifiable material in the unextracted bacterial lipids is lower and the percentage of fatty acids is higher than in the corresponding fractions of the unextracted non-bacterial lipids.   The differences between individual pairs of results are not large enough, because of the relatively small number of experiments, to admit of strict statistical analysis; but the fact that all of these results are in agreement endows them with some probable significance.

The data from these extraction experiments are in line with the widely accepted theory that the surface membrane of cells contains a large portion of unsaponifiable material.   It is indicated that an unsaponifiable-rich portion of the bacterial lipids, comprising about half the total, may be extracted, while a much larger portion of the non-bacterial lipids, containing the ordinary proportion of unsaponifiable material and fatty acids, is readily removed by ether.

CONCLUSIONS.

The foregoing experiments demonstrate that on the average not far from 40 per cent of the lipids excreted by dogs on a lipid-free diet is contained in the bodies of bacteria, or at least in a fraction of the feces estimated to be made up at least 90 per cent of bacteria. Most of the rest of the excretion is contained in the remaining solid fecal particles, all large enough to settle out of suspension readily

and estimated to be well over 90 per cent non-bacterial in nature. A negligible portion of the lipids is present in soluble or suspended form. These results indicate that the fecal lipid excretion is present almost entirely in cellular structures of one sort or another and does not represent an unabsorbed secretion or excretion into the intestine. However, there is also the possibility that the lipids are actually secreted into the intestine and then adsorbed by bacteria and other fecal particles. In fact, evidence that such is the case may be adduced from the failure to find any marked difference in the lipids of the bacterial and non-bacterial portions with respect to partition of unsaponifiable and fatty acid fractions and composition of fatty acids.

It is indicated by experiments in which the ease of extraction was studied that the bacterial lipids are less easily extracted by ether than the non-bacterial and that the portion of bacterial lipids which is extracted is richer in unsaponifiable material than the corresponding portion of non-bacterial lipids. The small extraction of lipids from dried bacteria by petroleum ether is in agreement with the first of these findings. It is questionable what bearing such results have on the problem at hand since it is possible, though not probable, that lipids might be adsorbed on bacteria more tenaciously than on other particles. It might be argued that adsorption can hardly explain the partition observed since the bacteria would probably present far more adsorbing surface than the other particles and should contain a larger portion of lipids; but this discrepancy might be explained by differences in nature of the adsorbing surfaces. In short there seems to be no experimental method available to determine between these possibilities. It seems more logical, however, to favor the view that a large portion at least of the excretion is contained in cells since practically all cells contain lipids and without doubt a large portion of the fecal solids, aside from the bone ash used as roughage, is made up of cells of various sorts.

Experiments similar to the above are being carried out on bile fistula dogs.

SUMMARY.

1. A modification of the Strasburger procedure for separation of bacteria from feces, applicable to large amounts, is described.

2. With this method the feces excreted by dogs on a lipid-free diet have been separated into portions made up very largely of (1) bacteria, (2) non-bacterial solids, and (3) soluble materials.

3. The lipid content of each of these fractions has been determined. In seven experiments the total fractions have been digested with strong alkali and the total unsaponifiable and fatty acid and solid and liquid fatty acid portions for each fraction determined. In nine experiments the bacterial and non-bacterial fractions have been extracted with ether before saponification; the ether extracts have been separated into neutral and free fatty acid portions; the neutral fractions have been saponified and separated into unsaponifiable and fatty acid portions; and the extracted suspensions have been digested with strong alkali, the lipids have been removed, weighed, and separated into unsaponifiable and fatty acid portions.

4. About 40 per cent, on the average, of the total excretion is contained in the bacterial fraction. Most of the remainder is contained in the non-bacterial solids, with a negligible amount dissolved or in suspension.

5. The results are in agreement with previous work in respect to partition of unsaponifiable material and fatty acids in the total lipid excretion, about 30 per cent being found to be unsaponifiable material and 60 per cent fatty acids, with a 10 per cent loss in separation.

6. Essentially the same partition of unsaponifiable material and fatty acids has been found for the bacterial and non-bacterial lipids; i.e., the same partition found in total lipids.

7. No significant difference in ratios of liquid to solid fatty acids has been made out in the bacterial and non-bacterial lipids, and, though there is considerable variation in the results, they are in fairly close agreement with previous work. ·

8. It is indicated that lipids are less easily extracted by ether from the bacterial than from the non-bacterial fraction and that the lipids extracted from the bacterial fraction are richer in unsaponifiable material and poorer in fatty acids than are the lipids extracted from the non-bacterial solids in which the partition is the same as in total lipids. The reverse is indicated for the unextracted lipids.

9. The results indicate that the fecal lipid excretion is com-

posed of lipids contained in various cellular structures such as bacteria, desquamated epithelial cells, protozoa, *etc.*, of the feces. The possibility cannot be ruled out at present, however, that the fecal lipids represent a secretion into the intestine which has been adsorbed in whole or part on solid particles of the feces.

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A NOTE ON THE DETERMINATION OF THE DIGESTIBILITY OF PROTEIN BY BERGEIM'S METHOD.*

BY WILLIS D. GALLUP.

(From the Department of Agricultural Chemistry Research, Oklahoma Agricultural Experiment Station, Stillwater.)

In 1926 Bergeim (1) suggested a modified procedure for determining the digestibility of food, which has for its unusual feature "the addition to the food of small amounts of iron oxide (or other suitable substance)." The ratio of the amount of iron to the amount of any food substance in the diet and in the feces is then determined and from this the percentage of utilization is calculated. In a recent study (2) of the digestibility of the proteins of some cottonseed products this method gave results which agreed closely with those obtained by the usual procedure which requires a measurement of the intake and output of nitrogen over a given period. Difficulty was encountered only in making a complete recovery of the iron, which, as pointed out, might have been due to the nature of the diets employed. More recently Heller, Breedlove, and Likely (3) have made a comparison of the two methods. Since then it has become necessary to determine the digestibility of food substances in diets containing as much as 1 per cent soluble iron salts, the presence of which was regarded as being unfavorable to the use of the modified procedure. To overcome this possible objection silica was used in place of iron oxide, as silica may be regarded as an insoluble compound and one that may be excreted with practical completeness in the feces.

It is the purpose of this paper to show the application of this method to the determination of the digestibility of the protein in a diet made up of natural foods and to compare it with the usual method, as well as the proposed method with iron oxide.

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The methods employed in conducting this study with albino rats were essentially the same as described in a previous paper (2). The diets were composed of corn 40 per cent, wheat 48 per cent, casein 8 per cent, NaCl 1 per cent, $CaCO_3$ 1 per cent, cod liver oil 2 per cent. The silica was prepared from clean, white sea sand which was ground to pass a 100 mesh sieve, and digested with dilute hydrochloric acid to remove soluble impurities. The finest particles were removed by decantation and the remaining silica used to make up approximately 1 per cent of the diet.

The determinations of silica in the food and feces were made on 5 gm. of food and 1 gm. of feces by the usual methods, except that that portion of the ash which was insoluble in hydrochloric acid was taken as silica. Such a procedure allowed for a number of determinations to be made rapidly and with surprising accuracy. The food or feces were ashed at low red heat. 2 cc. of 1:1 hydrochloric acid were added to the residue and the contents evaporated to dryness. After being heated at 110° for about 1 hour the residue was taken up with 2 cc. of concentrated hydrochloric acid, heated slightly, and 10 cc. of water added. The insoluble material was filtered off and ashed in weighed crucibles.

The procedure outlined by Bergeim (1) was followed for the iron determinations. A number of determinations were made on samples of food to which known amounts of silica and iron had been added. In a series of feeding experiments it was found that the amount of iron actually recovered in the feces during four feeding periods varied less than 10 per cent from the theoretical (assuming the elimination to be 100 per cent), while even closer agreement was obtained in the silica determinations during six feeding periods. It was calculated that if recovery of silica were only 85 per cent of the theoretical, the per cent digestibility would come within the limits of accuracy of the older method.

The important constituents of the diets used are as follows:

Diet No.	Protein.	Iron.	Silica.
	per cent	per cent	per cent
200	17.15		
201	16.98	0.122	
202	17.21		1.08
203	17.46		1.10

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The cod liver oil in the diets aided in keeping a uniform distribution of iron oxide and silica through the food mixture and no separation of these constituents from the other food particles could be detected by chemical analysis of the food residues and scattered food.

A comparison of the results obtained in this study has been tabulated in Table I.

TABLE I.
Results Obtained on Digestion Trials.

Diet No.	Period No	Digestibility coefficient.			
		Usual method	Iron method	Alternate method	Difference
200	1	86 55			
	2	87 24			
	3	89 22			
	4	88 96			
201	1	86 61	87 84		1 23
	2	86 57	88 22		1 65
	3	87 94	89 54		1 60
	4	88 02	88 90		0 88
202	1	88 58		88 28	0 30
	2	85 64		85 65	0 01
	3	88 38		87 92	0 46
203	1	86 22		86 30	0 08
	2	88 85		88 35	0 50

The above results without the additional amount of data collected during these feeding trials are taken as attesting to the accuracy of the method proposed.

CONCLUSION.

In carrying out digestibility studies by Bergeim's method with certain diets or under conditions which do not permit the use of iron oxide, silica in sufficient quantities may be used as a suitable substitute. When such a substitition is made, certain alterations in the procedure become necessary but the method still retains most of its desirable features.

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GUANIDINE STRUCTURE AND HYPOGLYCEMIA.

By FRITZ BISCHOFF, MELVILLE SAHYUN, and M. LOUISA LONG.

(From the Chemical Laboratory of the Potter Metabolic Clinic, Santa Barbara Cottage Hospital, Santa Barbara.)

INTRODUCTION.

By a series of structural changes, Frank, Nothmann, and Wagner (1) developed synthalin, the most powerful hypoglycemic synthetic product known today. The details of their researches are not available. The increased hypoglycemic activity of agmatine over guanidine offered the first clue as to the nature of the groups which were productive of hypoglycemic activity. It may be gathered from the recent patent literature (2) that the synthesis of synthalin was accidental, synthalin (diguanidinodecamethylene) forming when the preparation of the homologue of agmatine (monoguanyldiaminodecane) was attempted. The enthusiasm which greeted the announcement of synthalin as a "mouth insulin" was dispelled as soon as the nature of the physiological activity was brought to light. Blatherwick *et al.* (3) showed in this laboratory that by parenteral administration an acute nephritis is produced in rabbits, along with injury to the liver, as shown by a decreased ability to deaminize glycine. Clinicians observed the same effects in diabetic patients on a synthalin treatment. These results suggested that synthalin might cause hypoglycemia by preventing normal glyconeogenesis. Other profound differences in the action of synthalin and insulin were brought forth by Bodo and Marks (4). They showed that synthalin causes the liver to be depleted of glycogen and brings about a breakdown of muscle glycogen in addition to a disappearance of sugar. Simola (5) found that the inorganic phosphorus does not always decrease when synthalin produces hypoglycemia, a phenomenon so characteristic of insulin hypoglycemia.

If synthalin produces hypoglycemia solely by retarding deaminization and by depleting the liver glycogen, there is little promise of its being the stepping stone to the development of a more insulinlike hypoglycemic compound. However, Bodo and Marks' experiments upon perfused muscle and the experiments in this laboratory upon the influence of synthalin on the glucose toler-

ance, show an insulin-like action in the disappearance of the glucose These results tempt one to try the effect of modifying the synthalin structure. The problem has been attacked in this laboratory from two angles. One is to study further the physiological action of synthalin. This problem is complicated by the disagreement which still exists as to the mechanism of insulin hypoglycemia. Another attack is to study the effect of changes in guanidine structure.

In this paper the hypoglycemic properties of twenty-five guanidine compounds were studied. Blood urea and amino acid nitrogens were followed as an index of liver and kidney damage. The combined effect of the guanidine compound and adrenalin upon the blood sugar was determined in order to follow the mobilization and disappearance of glycogen. The lethal dose of the more toxic derivatives was established. The effect of several of the guanidine compounds upon the glucose and glycine tolerance is included. No new biochemical line of attack for elucidating the mechanism of hypoglycemia is presented. The criteria mentioned above must now be applied before any substance may be said to share the properties of insulin. The object of the study was primarily to follow the effect of structure upon the physiological action of the guanidine compound. In surveying the guanidine literature, it becomes at once apparent that wide gaps still exist for certain types of compounds. In this paper an attempt was made to study as many types as possible. The new compounds which were synthesized have been presented in another paper (6). The list of known aromatic guanidine compounds is very large because of their use as rubber accelerators. Unfortunately, as these studies show, the benzene nucleus is not productive of hypoglycemia. An exhaustive investigation for the aromatic series was, therefore, abandoned. In the aliphatic series the known derivatives are either basic compounds, acyl derivatives, or compounds potentially derivatives of guanidine formic acid or its homologues. Alcohol or ketonic derivatives are distinctly rare. Guanidine ethanol was studied by Alles (7). He made a study of nine simple guanidine derivatives, none of which showed any hypoglycemic effect. The study of isolated compounds, amyleneguanidine by Simonet and Tauret (8), acoin by Cannon (9), and creatine by Hill (10), have appeared. It has been rumored that the synthalin workers

tried out the action of 300 guanidine derivatives before they placed synthalin upon the market.   At the time their studies were made, the criteria applied by us had not been established.

Non-Toxic Compounds without Hypoglycemic Action.

Rabbits were found to tolerate the following guanidine compounds given intravenously in doses of 200 mg. or over per kilo without a visible toxic effect: dicyanodiamide, hydroxymethylcyanoguanidine, guanylurea, guanine, dicarbethoxy- and dithiocarbethoxyguanidine, creatinine, creatine, and arginine.   Ammelide and guanidine acetic acid were tried in doses of 100 mg. per kilo.   See Table I.   Blood sugar determinations were made at five intervals during the first 8 hours and at 25 hours.   The animals were fasted 24 hours previous to being dosed.   No drop in blood sugar was observed.   Since the results were negative, we have not included the 400 sugar values determined in this series. In no instance did the value drop more than 10 per cent from the initial value or rise more than 15 per cent.   Urea and amino acid nitrogens were determined for rabbits which had received over 200 mg. per kilo of dicyanodiamide, creatinine, guanylurea, hydroxymethylcyanoguanidine, and guanine.   There was no rise at the 5th or 25th hours.   See Table VI.

It is significant that these non-toxic guanidine compounds contain negative groups.   It will be noted that guanylurea and guanidine formic acid are the successive hydrolysis products of dicyanodiamide (if one accepts this compound as cyanoguanidine). Ammelide, dicarbethoxyguanidine, and dithiocarbethoxyguanidine may be regarded as derivatives of guanidine formic acid, so that all these compounds are more or less closely related.

Hill (10) has recently reported the hypoglycemic action of creatine when administered to dogs subcutaneously or by mouth. We were unable to duplicate his results with rabbits and suggest that an impurity in his creatine may have functioned as the hypoglycemic principle.   A similar experience was encountered by us in the case of hydroxymethylcyanoguanidine.   A sample of this compound was furnished us by the Jackson laboratory of E. I. du Pont de Nemours and Company, Inc.   It was marked "crude" and contained some dilute, acid-insoluble material.   Since the

TABLE I.

Guanidine Derivatives without Demonstrable Toxic or Hypoglycemic Effect.

Compound	Formula.	Doses without effect, mg base per kg.
Dicyano-diamide.	$\underset{NH}{\overset{NH}{\|\|}}\underset{}{}C-\underset{}{\overset{H}{N}}-CN$	100, 125, 300, 320
Hydroxy-methyl-cyano-guanidine.	$HO-\overset{H}{\underset{H}{C}}-\overset{H}{\underset{}{N}}-\overset{NH}{\underset{}{C}}\ N-CN$	75, 75, 100, 200, 300
Guanylurea.	$NH_2-CNH-\overset{H}{N}-CO-NH_2$	140, 200, 275
Guanine.	$NH_2-C\overset{NH-C=O}{\underset{N-C-N}{C-N-CH}}$	50, 200, 200
Ammelide.	$NH_2-C\overset{N=C-OH}{\underset{N-C-OH}{N}}$	100
Dicarbeth-oxyguan-idine.	$\overset{H}{N}=C\overset{NHCOO\ Et.}{\underset{NHCOO\ Et.}{}}$	20, 37, 60, 103, 200
Dithiocar-bethoxy-guanidine.	$HN=C\overset{NHCOS\ Et.}{\underset{NHCOS\ Et.}{}}$	60, 60, 170
Guanidine acetic acid.	$NH_2CNH-NHCH_2COOH$	125
Creatinine.	$HN-CNH-N\overset{O=C-\ ---CH_2}{\underset{CH_3}{}}$	340
Arginine.	$NH_2-CNH-NH(CH_2)_3-\overset{NH_2}{\underset{H}{C}}-COOH$	250, 270
Creatine.	$NH_2CNH-\overset{CH_3}{N}CH_2COOH$	100, 235, 300 100, 500 *per os.*

All compounds given parenterally unless otherwise specified.

product produced hypoglycemia without death in three trials, it was of unusual interest. Only a few gm. of the material were at our disposal. At our request, Dr. Powers of the du Pont Company kindly sent us the following information.

"Calcium cyanamide was bought from the American Cyanamide Company. Dicyanodiamide was obtained from the calcium salt by hydrolysis with boiling water, according to the method of Söll and Stutzer (*Ber. chem. Ges.*, **42**, 4533 (1909)). The recrystal-

TABLE II.

Blood Sugar Changes Following Injection of Hydroxymethylcyanoguanidine from Different Sources.

	Dose.	Administration.	Blood sugar in mg. per 100 cc.						
			Initial.	1 hr.	1½ hrs.	2 hrs.	3 hrs.	4 hrs.	8 hrs
	mg. per kg.								
Power's product.	45	Intravenous.	87			53		95	118
	80	Subcutaneous.	121			133	148	143	114
	60	Intravenous.	111	74		125		125	
	40	"	105	42	47	67	111	125	
Bischoff's product.	75	"	118	91	105		111	91	
	75	"	100	100	95	103		95	
	100	"	105		103		114	108	
	200	"	118	114		118		120	
Denatured product.	120	Subcutaneous.	114			160		200*	
	120	"	100		154			295	334*

* Indicates death.

lized dicyanodiamide was heated with a formaldehyde solution according to the method of Pohl (*J. prakt. Chem.*, **77**, 537 (1908))."

We then synthesized the compound according to the directions of Pohl. The product obtained by us was without hypoglycemic action. Since hydroxymethylcyanoguanidine is not stable in aqueous solution at higher temperatures, a deliberate attempt to reproduce the hypoglycemic impurity by refluxing dicyanodiamide with formalin for many hours was made. The product so obtained was toxic, producing hyperglycemia and death. No attempt was made to fractionate the resin. The results of the experiments with hydroxymethylcyanoguanidine are recorded in

TABLE III.

Action of Aromatic Guanidine Compounds.

Compound	Dose of base (mg. per kg.)	Administration		Blood findings in mg. per 100 cc.					
				Initial	1¼ hrs.	5 hrs.	8 hrs.	24 hrs.	64 hrs.
Triphenylguanidine.	10	Intravenous.		*†					
	30	"		*†					
Tri-o-tolylguanidine.	40	Subcutaneous.	Sugar.	95	105*				
	34	"	Sugar.	95	98†	100 †		105	
			Amino N. "	10.5		10.0		10.3	
			Urea "	23		22.5		26.5	
Di-p-tolylguanidine.	25	Subcutaneous.	Sugar.	95	98†	100 †		105	
			Amino N. "	10.5		10.0		10.3	
			Urea "	23		22.5		26.5	
Dibenzoylguanidine.	50	Subcutaneous.	Sugar.	100 *	133*				
	75	"	Sugar.		103	105		100	
	60	"	Sugar.	100		10 0		9.8	
				10 3		22 5		21.0	
				21.5					
Guanyl-p-amino dimethylaniline.	200	Subcutaneous.	Sugar.	105	45*†				
	225	"	Sugar.	118	111†	167 †	87†	105 *	
	100	Intravenous.	Sugar.	111	40*†	167	167	100	*
	100	"	Sugar.	105	154	154 †		100	
	100	"	Sugar.	105	157†				
			Amino N. "	10.5		10.5		10.0	
			Urea "	27.5		28.0		46	

Guanylbenzidine.	200	Per os.	Sugar.	105	98	114		125	40 *†
	100	Per os.	Amino N. "	85	89	74	74	77	20.0
	75	Intravenous.	Urea "	100	69	114		*	24.2
			Sugar. "						

* Indicates death.

† Indicates nervous symptoms, asphyxiation, convulsions.

When these symbols are placed in the column headed initial, they indicate the effect a few minutes after dosing.

Table II.   Guanidine compounds which produce hypoglycemia
without death are a distinct rarity and we feel it a great loss to this
study that the impurity could not be isolated.

Aromatic Compounds.

Alles (7) observed that diphenylguanidine was more toxic than
methyl-, dimethyl-, and ethylguanidine.   He found the minimum
lethal dose slightly greater for triphenylguanidine.   We have
studied the action of six aromatic guanidine compounds: triphenyl-
guanidine, tri-o-tolylguanidine, di-p-tolylguanidine, dibenzoyl-
guanidine, guanyl-p-dimethylaminoaniline, and guanylbenzidine.
See Table III.   Alles' toxicity experiments were performed upon
rats.   As all of our compounds were tried upon rabbits, we have
tested out one of his compounds, triphenylguanidine, in order to
make our results comparable with his.

None of these compounds showed any unusual hypoglycemic
properties, and one might postulate that the benzene nucleus was
not productive of hypoglycemia.   In the aliphatic compounds
studied, hypoglycemia ran parallel with the lethal dose.   In the
case of triphenyl-, tri-o-tolyl-, and di-p-tolylguanidine, death
occurs without hypoglycemia.   Guanyl-p-dimethylaminoaniline
produced a distinct hypoglycemia with convulsions in one instance
on a 100 mg. per kilo dose.   In three other cases a hyperglycemia
with subsequent death resulted.   Guanylbenzidine produced a
hypoglycemia in several instances on the 100 mg. dose.   The
increased hypoglycemic effect by lengthening the carbon chain
is not demonstrated in these two aromatic compounds.   It is
interesting to note, however, that guanyl-p-dimethylaminoaniline
is less toxic than its parent compound, p-dimethylaminoaniline.
This is in distinct contrast to diguanyldiaminopentane, which is
toxic, while the parent diaminopentane is harmless.

In guanylbenzidine the carbon chain is of approximately the
same length as synthalin.   This aromatic compound was ob-
served to be active by mouth in the rabbit, while synthalin has
never been observed to be active by mouth in this animal.   The
effect of the benzoyl group is very similar to the acetyl group,
dibenzoylguanidine being less toxic than the phenyl and sub-
stituted phenyl derivatives.   An aromatic guanidine compound

with hypoglycemic properties has recently been reported under the name of acoin. This derivative is a tri-substituted guanidine in which two of the groups are anisole and one is ethylphenyl ether. 500 mg. per kilo of this substance produces hypoglycemia in 1½ to 2 hours with toxic symptoms. Since this compound is less active even than guanidine, it is of little interest in the study of insulin hypoglycemia.

Triphenylguanidine is the most toxic aromatic compound studied, its lethal dose being of the same order as synthalin. The inherent toxic effect of the aromatic compounds is quite distinct from the aliphatic series. Convulsions and nervous symptoms follow without hypoglycemia. No increase in the urea or amino acid nitrogens was found for tri-o-tolylguanidine or di-p-tolylguanidine in doses which produced continued convulsions for hours.

Blood calcium was determined for two rabbits which had received di-p-tolylguanidine and were in tetany-like convulsions. The values obtained, 13.9 and 13.8, were perfectly normal, however.

Synthalin Homologues.

In comparing the action of diguanidinopentamethylene, diguanidinooctamethylene, diguanidinodecamethylene (synthalin), and diguanidinododecamethylene, one has the opportunity of studying the effect of lengthening a hydrocarbon chain in a homologous series. If these compounds have a true insulin-like action in addition to their toxic effect (retarding effect upon deaminization, failure to respond to adrenalin), one might expect a quantitative difference in the relation of the two effects as one progresses from one compound to another. Our results show no such difference. When death occurs, it is accompanied by hypoglycemia. In isolated cases, hypoglycemia is produced without death. If the animal does not die within the first 5 hours, an increase of urea and amino acid nitrogens always occurs.

Our experiments show that the toxic and hypoglycemic effects of the synthalin series increase with the lengthening of the carbon chain. The maximum effect is reached in synthalin, neosynthalin being less powerful. From our data, the lethal doses for rabbits in mg. of base per kilo given parenterally may be evaluated as follows: pentamethylene, 50; octamethylene, 7; decamethylene, 4; and

dodecamethylene, 8. See Table IV. Individual variation for animals is more apparent where the dose is larger. ⸱An occasional animal survived 70 mg. of the pentamethylene derivative.

TABLE IV.

Establishing the Minimum Hypoglycemic Dose for Diguanidinopenta- and Diguanidinooctamethylene and for Guanylpiperidine and Isoamylguanidine.

Compound.	Administration.	Dose.	Blood sugar in mg. per 100 cc.						
			Initial	1 hr.	1½ hrs.	3 hrs.	5 hrs.	8 hrs.	24 hrs.
		mg. per kg.							
Diguanidino-pentamethylene.	Subcutaneous.	40	95		83	87	100	125	108
	Intravenous.	40	140		125	91	118	130	
	"	50	98		55	40	35	35	*
	Subcutaneous.	50	100		133	108	45		
	Intravenous.	58	105		91	65	45		38*
	"	65	95		80		67	74	67*
	Per os.	300	87		85	74	74	95	91
	" "	600	100		125	125	87		43*
Diguanidino-octamethylene.	Intravenous.	3.5	111		105	108	105	111	
	"	3.5	80		80	100	85	125	
	"	5.0	100		125	57	61	58	*
	"	7.0	108		76	24	20*		
	"	7.0	91		74	50	31	*	
	"	7.0	100		40		25*		
	"	8.8	100		66	43*			
	Subcutaneous.	17.0	114		62	31	*		
Guanylpiperidine.	Intravenous.	30	95	98	105		105		100
	"	60	95	35	66		125		
	Subcutaneous.	72	105	100	95	93	129		100
	Intravenous.	72	100	40	*				
	"	72	100	35	*				
	"	75	118	50		114	118		100
Isoamylguanidine.	"	35	91		93	93	91		100
	Subcutaneous.	70	91		100	118	114		98
	"	70	105		91	100	100		100
	"	105	108		93	32	*		
	Intravenous.	105	105		74	68	111		118

' Indicates death.

Hypoglycemia without ensuing death was encountered more times with the pentamethylene derivative than with the others

in the series. This was possibly due to the fact that a finer graduation of dosing was accomplished. The rise in urea and amino acid nitrogens, the low values for liver and muscle glycogen, the failure of the animal to respond to adrenalin, and the liver and kidney damage, as shown by microscopic sections, following the administration of a hypoglycemic dose, ran strictly parallel to the action of synthalin.

Blatherwick *et al.* (3) observed that synthalin has a variable influence on the glucose tolerance of rabbits, a hyperglycemia resulting in some cases, while in others the glucose appeared to be completely utilized or stored. Similar results were obtained by us for the pentamethylene and octamethylene derivatives.

TABLE V.

Effect of Cyanamide upon Blood Sugar.

Dose.	Blood sugar in mg. per 100 cc.										Remarks.
	Initial	1 hr.	1½ hrs.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	6 hrs.	7 hrs.	8 hrs.	
mg. per kg.											
44	95	87	114	143							
100	95	160	200		308	276			210	150	
100	100	91		125	200		216				2 units insulin per kilo 3rd hr.
100	105	222		266	320	133	114				4 units insulin per kilo 3rd hr.

Cyanamide.

Since cyanamide may be regarded as the parent of guanidine derivatives and since it reacts directly at room temperature with many amino compounds to form guanidines, it was of interest to test out its effect upon the blood sugar. The rabbit was found to tolerate 100 mg. per kilo of the substance given intravenously. Transient nervous symptoms and weakness developed. The blood sugar rises to unusual heights. The hyperglycemia may be counteracted by insulin. The results are given in Table V.

Amino Acid and Urea Nitrogens.

The changes of blood amino acid and urea nitrogens, following the injection of seventeen of the guanidine derivatives, have been

TABLE VI.

Changes in Blood Urea and Amino Acid Nitrogen Following Injections of Guanidine Compounds.

Compound.	Dose.		Initial.	1 hr.	1½ hrs.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	6 hrs.	7 hrs.	9 hrs.	24 hrs.	28 hrs.	Remarks.
	mg. per kg.					Blood findings in mg. per 100 cc.									
Diguanidino-pentamethylene.	90	Sugar.	105	98		100		58		40		35	*		Subcutaneously.
		Amino N. "	10.0									33			
		Urea "	23												
	70	Sugar.	95	74		80		50		40.5		74	67		
		Amino N. "	10					23		67			26.5		
		Urea "	20					37.5					55.5		
	600	Sugar.	100	125			125	114		87			69	43	Per os.
		Amino N. "	9.5				9.8							53.5	
		Urea "	20.0				20.8							30	
	65	Sugar.	105			82		42							
		Amino N. "	10.3					16.3							
		Urea "	25					26							
Diguanidino-octamethylene.	17	Sugar.	114			62			31	*					Glucose tolerance 2nd hr. No urine sugar.
		Amino N. "	10.8			13.5			29.8						
		Urea "	17			17.5			23.5						
	7	Sugar.	114			118	71		58	167		175	71		Glucose tolerance 5th hr.
		Amino N. "	13.8			14.0	14.5		17.3	200			35		
		Urea "	27			29.5	34.5		36.0			62.5			
	3.5	Sugar.	80			80	100		85			160	105		Glycine tolerance.
		Amino N. "	13.5			40.5	39.5		38	28.5		29	30		
		Urea "	28			33			50			53	108		

Compound	Dose											Remarks
Neosynthalin.	4	Sugar	103	50	40	121	174	185	125		114	Glycine tolerance.
		Amino N. "	12.3			44.5	39.4	24.3	15.5		11.2	
		Urea	27.8			34.7	36.5	51.5	55.5		68.2	
	8	Sugar	111	95 †	95 †	100	138	200	200		148	Glycine tolerance.
		Amino N. "	11.8			40.5	37.2	25.8	21.5		11.8	
		Urea	24.5			32.0	31.5	47.0	60.0		68	
Guanyl- piperidine.	72	Sugar	114	95	95	74	240	121	129		87	
		Amino N. "	10.7				10.5	13.8	8.7		10.2	
		Urea	28.5					47.5	28.2		37.5	
	72	Sugar	105	50		143	93				100	
		Amino N. "	10.8	11.8							11.4	
		Urea	31.5								25.8	
	75	Sugar	118	100		114			118		100	Subcutaneously.
		Amino N. "	9.4						41.5		11.4	
		Urea	26.5								25.8	
	72	Sugar	105			91					100	Glycine tolerance.
		Amino N. "	10.5								10.0	
		Urea	27								27	
	40	Sugar	114	133	47			200	125	125	118	
		Amino N. "	10		28.5			31	23	23	12.5	
		Urea	23.8					37	42	42	33.5	
Guanyl- piperazine.	140	Sugar	95	133 †		240					55	*
		Amino N. "	10.8								9.5	
		Urea	24								41	
	140	Sugar	111	132		167	250				125	*
		Amino N. "	9.8			10.5	9.8				7.5	
		Urea	25				32.5				115	
	100	Sugar	112	111		118		111		90	154	Subcutaneously. *
		Amino N. "	10.0							8.4	9.8	
		Urea	27.5							34.5	50.0	

TABLE VI—*Concluded.*

Compound.	Dose.		Initial.	1 hr.	1½ hrs.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	6 hrs.	7 hrs.	9 hrs.	24 hrs.	28 hrs.	Remarks.
	mg. per kg.														
Isoamyl-guanidine.	70	Sugar.	105		91		100		100		100				
		Amino N. "	11.4				9.8		10.3		9.3				
		Urea "	32.5				37.5		45.5		38.5				
	90	Sugar.	111			†	125		450		125				
		Amino N. "	9.5				9.1		8.2		7.5				
		Urea "	35.5				38.5		47.5		64.5				
Dicyano-diamide.	320	Sugar.	100		95			87	95				103		
		Amino N. "	9						9.3				8.9		
		Urea "	30						29.5				30		
Creatinine.	340	Sugar.	90		100			103	95				100		
		Amino N. "	8.4						7.5				7.0		
		Urea "	27.5						27.2				28.0		
Guanylurea.	200	Sugar.	95		105			105	95				105		
		Amino N. "	9.5						9.3				9.5		
		Urea "	27						26.5				26.5		
Hydroxy-methylcyan-oguanidine.	300	Sugar.	91		108			111	105				100		
		Amino N. "	10.0						9.8				9.5		
		Urea "	23						23				22.5		

Blood findings in mg. per 100 cc.

Guanine.	200	Sugar.	100	103			95		100	Subcutaneously.
		Amino N. "	9 5				9 2		9 5	
		Urea "	25 5				26		26 5	
Creatine.	235	Sugar.	100	111		100	105		100	Subcutaneously.
		Amino N. "	10 5			9 5	8 5		8 9	
		Urea "	27			25 5	29 0		24 0	

All compounds given intravenously unless otherwise noted. In the glycine tolerance, 1 gm. per kilo given subcutaneously.

* Indicates death.

† Indicates 1 mg. of adrenalin given subcutaneously.

followed. These are summarized in Table VI. For the aromatic compounds see Table III. It has been pointed out in another part of the paper that the non-toxic guanidine compounds, those showing no toxic symptoms upon doses of 200 to 300 mg. of compound per kilo, show no change either in the amino acid or urea nitrogens. In the series of the homologues of synthalin, both of these values always increase materially if the animal shows hypoglycemia and lives 5 hours. In several instances the augmentation of these values was shown in less time. The increase in the urea nitrogen without hypoglycemia and without an increase in the amino acid nitrogen has been observed for three compounds: guanylpiperazine, guanyl-p-dimethylaminoaniline, and isoamylguanidine. Two aromatic compounds, tri-o-tolyl- and di-p-tolylguanidine, showed no change in the nitrogen values with doses which were extremely toxic.

Guanylpiperidine, like the synthalin homologues, shows a diminished tolerance to glycine. One experiment is recorded in Table VI. It will be noted that there is a marked rise in amino acid nitrogen at $1\frac{1}{2}$ hours with high values through the 7th hour. The administration of glycine was found to increase the lethal dose of guanylpiperidine greatly. A series of experiments was attempted, but the animal invariably died before the data could be taken. The experiment recorded is one in which a subhypoglycemic dose was given. In experiments in which no glycine was given the rise in amino acid nitrogen is considerably less than it would be for a synthalin homologue, returning to normal at 24 hours.

A marked hyperglycemia was observed in the glycine tolerance experiments with guanylpiperidine, neosynthalin, and diguanidinooctamethylene. Blatherwick et al. (3) obtained a mild hyperglycemia for their control glycine tolerance animals and Bodo and Marks (4) a preliminary hyperglycemia in their synthalin series. Our observations indicate a combined effect.

Our results may be summarized as follows: The type of guanidine poisoning, characterized by tetany-like convulsions, dyspnea, and prostration, and brought about by the aromatic derivatives is not connected with liver or kidney damage. Kidney damage is brought about by either the aliphatic or aromatic guanidine bases. It does not appear to be connected with the phenomenon of hypoglycemia. Liver damage, on the other hand, runs parallel with

hypoglycemia.   Blatherwick *et al.* (3) showed that the glycine tolerance curve was normal for animals with spontaneous or experimental nephritis, so that an increase in amino acid nitrogen should not be interpreted as possible renal damage, the urea nitrogen alone being an index of such damage.

Fate of the Glycogen.

Glycogen determinations for the liver and muscle of two rabbits which were in guanidine hypoglycemia were obtained.   One of these animals received 58 mg. of diguanidinopentamethylene per kilo.   It was killed the 3rd hour, when the blood sugar had fallen to 61.   Upon analysis the liver gave 18 mg. of glycogen per 100 gm., the muscle 60 mg. per 100 gm.   A control rabbit on the same dose lived 24 hours, the blood sugar falling progressively until it reached 38 the 24th hour.   The other rabbit received 72 mg. of guanylpiperidine per kilo.   It was killed 1 hour after dosing, when the blood sugar was 40.   The liver contained a trace of glycogen, the muscle 33 mg. per 100 gm.   These results are essentially the same as those obtained by Bodo and Marks (4) for synthalin.   These authors concluded that hypoglycemia follows the depletion of liver glycogen.   They were under the impression that the failure to respond to adrenalin, shown by an increase in blood sugar, was a result of the low glycogen content in the liver.   Cori and Cori (11) have recently shown in rats (and their observation has been confirmed in this laboratory on rabbits) that adrenalin, rather than depleting the liver of glycogen, actually increases the amount.   It will be noted in our two experiments that the muscle contains the same amount of glycogen as the blood contains glucose, while the liver is virtually depleted of glycogen.   In Table VII are recorded the experiments in which adrenalin was administered to the animal during the action of the guanidine compound.   The results for diguanidinooctamethylene are strictly comparable to those of Bodo and Marks for synthalin.   When hypoglycemia has once begun, adrenalin is not able to produce hyperglycemia.   The single observations for diguanidinopentamethylene and dibenzoylguanidine are analogous.   In the case of guanylpiperidine, however, two instances of hyperglycemia following hypoglycemia were observed. In the case of isoamylguanidine the adrenalin was given after the

blood sugar had returned to normal. No appreciable effect is noted. It will be recalled that in the experiments with the du Pont hydroxymethylcyanoguanidine, hypoglycemia without death was observed several times. Like guanylpiperidine and insulin,

TABLE VII.

Effect of Adrenalin upon the Blood Sugar of Animals in Guanidine Hypoglycemia

Compound.	Dose.	Blood sugar in mg. per 100 cc.										Time of death.
		Initial.	1 hr.	1½ hrs.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	6 hrs.	7 hrs.	8 hrs.	
	mg. per kg.											
Diguanidino-octamethylene.	3 5	111		105		108		108	*	111	148	
	5 0	100	125		80*	57		61	58			7th hr.
	7 0	100			40*		25					5th "
	7 0	91	74		50*	31	28					5th "
	7 0	108		76		40	24*	20				6th "
Diguanidino-pentamethylene.	5 8	105			91	65*		45			45†	
Isoamyl-guanidine.	100	105		74		68		111*			118	
Dibenzoyl-guanidine.	200	105	95*	45								2nd hr.
Guanyl-piperidine.	95	91	38*		50							3rd "
	72	95	63	*		60		125				
	72	87		61*	125	266	360					
	72	114	50	40*	74		121					
	72	105	95	95*	143	240						

* Indicates subcutaneous injection of 1 mg. of adrenalin.

† 24th hour blood sugar level, 38.

the maximum hypoglycemia was within 2 hours after dosing. We are unable to offer any explanation for the difference observed in the action of these compounds.

These results suggest that Bodo and Marks' observation should be interpreted in another way. The failure to respond to adrenalin

is due to a depletion of carbohydrate in reserves other than the liver.

Glucose Tolerance.

Since guanylpiperidine resembled insulin in the time required to produce hypoglycemia, a property shared by none of the other hypoglycemic guanidine compounds, the effect of guanylpiperidine upon the glucose tolerance was studied. Blatherwick *et al.* (3) obtained variable results with synthalin. We made observations similar to theirs for diguanidinopenta- and diguanidinooctamethylene. A rabbit which was in hypoglycemia through diguanidinooctamethylene and showed a blood sugar of 58 the 5th hour, was given a glucose tolerance. A normal glucose tolerance curve was

TABLE VIII.

Effect of Guanylpiperidine upon Glucose Tolerance.

Rabbit No.	Dose of guanidine compound.	Glucose in mg. per 100 cc. blood.						
		Initial.	1 hr.	1½ hrs.	2 hrs.	3 hrs.	5 hrs.	7 hrs.
	mg. per kg.							
B		91	166	182	170	114	105	95
B	75	108	182	182	206	210	138	118
11		100	150	140	122	105	100	110
11	75	93	129	118	125	105	105	105

In each case 1.75 mg. of glucose per kilo were given initially by stomach tube.

obtained. See Table VI. 16 hours later the animal again showed hypoglycemia. Another rabbit, which had received double the dose of the guanidine base, was given 2 gm. of glucose subcutaneously the 2nd hour, when its blood sugar was 62. 3 hours later the blood sugar had fallen to 31. The urine was sugar-free. A rabbit which received 70 mg. per kilo of diguanidinopentamethylene, showed hypoglycemia the 6th hour, when the blood sugar was 62. During the following 18 hours the blood sugar remained at this level. The animal was comatose. 4 gm. of glucose were then given subcutaneously. 26 hours later the blood sugar was 46. 4 gm. of glucose were given. The blood sugar rose to 74

3 hours later when the animal died.   In Table VIII are given the results of the effect of guanylpiperidine upon the glucose tolerance. Two rabbits were fasted 24 hours and given 1.75 gm. per kilo of glucose by stomach tube.   A control sugar curve was obtained. The rabbits were then rested a week.   The glucose tolerance was repeated with the simultaneous administration of 75 mg. per kilo of guanylpiperidine intravenously.   In one instance, the rise was definitely prolonged; in the other, a lowering is recorded.   Guanylpiperidine does not appear to differ markedly from the synthalin series in respect to the glucose tolerance.

Miscellaneous Experiments.

Bodo and Marks (4) showed that in perfused muscle, synthalin brought about the conversion of the carbohydrate to lactic acid. During hypoglycemia the oxygen consumption fell and the carbon dioxide rose.   Such a pronounced change in metabolism should affect the CO_2-combining power.   We followed the combining power in a rabbit which had received 50 mg. of diguanidinopentamethylene per kilo.   The initial value was 42.4 volumes per cent.   The 2nd hour the value was 43.3 with a blood sugar of 55. The 4th hour it fell to 39.5, the blood sugar being 35.   The 6th hour the combining power returned to the initial value.   The animal died in hypoglycemic convulsions the 8th hour.

Simola (5) observed that synthalin may or may not decrease the inorganic phosphorus during hypoglycemia.   In four cases observed by us there was no decrease and in two instances a very definite increase.   For guanylpiperidine the values rose from 4.45 to 5.31 and from 5.05 to 5.76 at the time of maximum hypoglycemia.   In an instance of isoamylguanidine hypoglycemia, the phosphorus increased progressively from the initial value of 2.85 to 6.25 the 5th hour.   In this instance a blood sugar of 68 was observed the 3rd hour.   At 5 hours the blood sugar was normal.   Diguanidinopentamethylene raised the phosphorus to 8.5 in 24 hours.   In this animal there was a progressive hypoglycemia from the 3rd to the 24th hours.   The results emphasize the distinct differences between the action of insulin and guanidine hypoglycemia.

Effect of Structure.

The synthalin workers regard the structure $H_2N—C—N—$ $\overset{NH}{\overset{\|}{}}$ $\overset{}{H}$

$(CH_2)_n—N\Big\langle$ as the nucleus in the development of their hypoglycemic guanidine compounds. Agmatine (aminobutylguanidine) was the first compound studied. It produces hypoglycemia in rabbits in 100 mg. per kilo doses. By lengthening the hydrocarbon chains or converting the amino group to a guanidine group the hypoglycemic activity was increased. Our results with isoamylguanidine and guanylpiperidine indicate that the amino group in agmatine is of little influence upon the hypoglycemic activity. Isoamylguanidine and isoamyleneguanidine have the same power as agmatine. Guanylpiperidine and diguanidinopentamethylene are about twice as active. Dicyanodiamide, hydroxymethylcyanoguanidine, guanylurea, guanine, ammelide, dicarbethoxyguanidine, dithiocarbethoxyguanidine, guanidine acetic acid, creatinine, creatine, and arginine were found to be nontoxic. There was no hypoglycemic effect or rise in amino acid and urea nitrogens. There were no general toxic symptoms. These compounds may be regarded as derivatives of guanidine formic acid or its homologues ($NH_2—C—NH—C—$ or $\overset{NH}{\overset{\|}{}}$ $\overset{O}{\overset{\|}{}}$

$NH_2—C$ $NH(CH)_x$ $C=O$). $\overset{NH}{\overset{\|}{}}$

It should be emphasized that the introduction of a negative group, while it may decrease the toxicity, does not render the derivative harmless. Acetyl- and dibenzoylguanidine are examples. An impure acetyl diguanidinooctamethylene, which we prepared, was also found to be less toxic than the parent base. When the guanidine nucleus is joined to an organic acid through carbon, it appears to be harmless; when it is joined through oxygen, the toxicity is decreased. The explanation of this difference is undoubtedly connected with the stability of the two classes of derivatives.

Triphenylguanidine, tri-o-tolylguanidine, di-p-tolylguanidine, dibenzoylguanidine, guanyl-p-aminodimethylaniline, guanylbenzidine, diguanidinopenta-, diguanidinoocta-, diguanidinodeca-, and diguanidinododecamethylene, guanylpiperidine, guanylpiperazine, and isoamylguanidine are toxic. All these derivatives are distinctly basic. None of them is amphoteric or upon hydrolysis yields amphoteric derivatives.

Guanylpiperidine, isoamylguanidine, isoamyleneguanidine, and guanylpiperazine are more or less closely related structurally. Isoamylguanidine and isoamyleneguanidine possess about half the hypoglycemic activity of diguanidinopentamethylene. Their action is similar. Guanylpiperidine appears to function in a slightly different manner. This has been pointed out elsewhere. Whether this difference may be attributed to the cycle or to unsymmetrical di-substitution cannot be decided. We rather expected guanylpiperazine to possess more powerful hypoglycemic properties than the experiments indicated.

EXPERIMENTAL.

Compounds.

Dicyanodiamide was prepared from Eastman calcium cyanamide by the method of Stöll and Stutzer (12). The m.p. was 204–205°. Guanidine acetic acid was also prepared by their method in which guanylurea is heated with monochloroacetic acid.

24.6 per cent N, theory; 24.8 per cent N for $CN_3H_4CH_2COONa$

Guanylurea sulfate was prepared by the hydrolysis of dicyanodiamide with dilute sulfuric acid.

27.7 per cent N, theory; 28.0 per cent for $CN_3H_4CONH_2 \cdot H_2SO_4$

Creatine, creatinine, and guanine were Eastman products. Dicarbethoxyguanidine and dithiocarbethoxyguanidine and the aromatic compounds were du Pont products. Hydroxymethylcyanoguanidine was prepared by the method of Pohl. 8.5 gm. of dicyanodiamide were dissolved in 50 cc. of water. The solution was heated to 80°. 7.0 gm. of formalin were added. The solution

was allowed to evaporate by standing in a current of air. The residue was crystallized from alcohol. The first crop of crystals when analyzed gave 50.8 per cent N by Kjeldahl procedure, the second crop 52.3 per cent N (theory 49.2 per cent). Pohl obtained a melting point of 118°. Our product did not melt below 138°. It began to soften at 120°.

The synthesis of diguanidinopenta- and diguanidinooctamethylene, guanyl piperidine, guanylpiperazine, the impure guanylbenzidine, and guanyl-p-aminodimethylaniline has been reported (6). The compounds described in this paper were used in these studies. Isoamylguanidine was prepared according to the method of Schenck and Kirchhof (13). Eastman isoamylamine was used as the starting material.

We have referred to neosynthalin as diguanidinododecamethylene. Analysis of the chloroplatinate gave values which checked with the theoretical value for a compound of that structure.

Analysis.
$(CN_3H_4)_2C_{12}H_{24}H_2PtCl_6$.
Calculated. 12 05 per cent N, 28.0 per cent Pt.
Found. 12 2 " " " (Dumas), 27 9 " " "

The melting points of the picrate, chloroaurate, and chloroplatinate have appeared in another paper (14).

Methods.

Rabbits, which had fasted for 24 hours were taken. Some of these animals had been used for standardizing insulin. The methods of Folin and Wu were used for the determination of sugar, urea nitrogen, and amino acid nitrogen. Pflüger's method was used for glycogen analysis and Fiske and Subbarow's method for inorganic phosphorus. The aqueous solutions of the various guanidine compounds injected were adjusted to neutrality. The volume of solution was adjusted so that not more than 10 cc. of solution was given. In the case of guanine it was not possible to prepare a solution of sufficient concentration. This compound was given subcutaneously as a suspension. In the glycine tolerance experiments 1 gm. of glycine per kilo was given subcutaneously. In the glucose tolerance experiments 1.75 gm. per kilo were given by stomach tube.

SUMMARY.

A comparative study of derivatives of guanidine formic acid and homologues, of aromatic guanidine bases, of synthalin homologues, and other aliphatic guanidine bases is reported.

The substitution of negative groups or aromatic nuclei is not productive of hypoglycemia.   The basic derivatives with long aliphatic side chains have the most powerful hypoglycemic action. The derivatives of guanidine formic acid and its homologues are non-toxic.

Hypoglycemic activity, lethal dose, and liver damage run nearly parallel.

In all the derivatives but guanylpiperidine, the glycogen reserve of the body is almost depleted before hypoglycemia.   Guanylpiperidine differs from the other derivatives in that hypoglycemia occurs the 1st hour, if it occurs at all.   In this respect it resembles insulin.

Guanylpiperidine appears to be slightly less toxic to the liver on a hypoglycemic dose than the other derivatives studied.

The toxic effect of the aromatic derivatives, shown by impaired respiration and nervous symptoms, and the kidney damage as shown by a number of derivatives both aliphatic and aromatic, do not appear to be connected with the hypoglycemic activity. The liver damage, on the contrary, is definitely involved.

The study was begun with the idea of finding a derivative which was more insulin-like than synthalin.   While guanylpiperidine could not be recommended for clinical trial, its physiological action is encouraging to the development of other derivatives. Since the guanidine derivatives have definitely been shown to produce hypoglycemia by mechanisms other than glyconeogenesis, particularly the depletion of the carbohydrate stores outside the liver, there is still a possibility that they are chemically related to insulin.

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A MICRO METHOD FOR THE ESTIMATION OF TOTAL CREATININE IN MUSCLE.

BY SEVERO OCHOA AND JOSÉ G. VALDECASAS.

(From the Physiological Laboratories of the Junta para Ampliación de Estudios and the Faculty of Medicine, University of Madrid, Spain.)

The available methods for tissue creatine require much more material than can usually be obtained from small animals. Moreover, in the course of an observation on the changes of muscle creatine under different experimental conditions a method is needed with which creatine can be estimated in so small an amount of tissue as to allow a series of estimations to be carried out on the same animal.

Our efforts were directed to finding a suitable method of estimating creatine if possible in as small an amount of muscle as a few mg.

As a result of a long series of experiments a method was devised with which it was rendered possible to estimate creatine in amounts of muscle from 5 to 100 mg. (1). Since then our work has been considerably extended and a quantity of experimental data has been accumulated leading to some slight modifications in the original technique which we think render it more accurate.

The method finally adopted is as follows:

Small fragments of muscle are removed as promptly as possible and dropped into previously weighed Erlenmeyer flasks of about 50 cc. capacity. The flasks are kept tightly closed to prevent evaporation of moisture until they have been weighed again.

The weighings should be made with a precision balance, and care taken to determine 0.1 mg. differences in weight. A torsion balance accurate to 0.1 mg. is to be preferred as the weighing can be carried out much more quickly and with the same degree of accuracy.

Muscle can be removed from living animals under anesthesia,

care being taken in this case that the samples are not too much impregnated with blood. In any case the weight of muscle removed should not be less than 5 mg. nor greater than 100 mg. As a rule samples averaging 20 to 50 mg. should always be taken.

The weighed muscle is treated with 0.2 cc. of 0.2 N HCl for each mg. in weight (standardized pipettes graduated in 0.01 cc. must be used). The flasks are next covered with tin-foil and heated in the autoclave to 120° for 25 minutes.

After cooling, 0.2 cc. of pure 1.2 per cent picric acid for each mg. of muscle is added, and the contents mixed and allowed to stand for 5 minutes. The abundant protein precipitate which settles down is now separated by filtering.

A fixed volume of fluid is pipetted off from the clear filtrate and transferred to a flask, then 0.5 volume of 5 per cent NaOH is added, and the contents mixed. After standing for 5 to 8 minutes the color comparison is made as usual against a standard made in the following way.

To 5 cc. of a 0.002 per cent creatinine solution in 0.2 N HCl, 5 cc. of pure 1.2 per cent picric acid and 5 cc. of 5 per cent NaOH are added. After this has been mixed and has stood for 5 to 8 minutes the standard is ready for use.

The height of the standard (10 or 20 mm.) divided by the reading of the unknown and multiplied by 400, gives the total creatinine in mg. per 100 gm. of muscle.

DISCUSSION.

For transforming creatine into creatinine we adopted the method of acid disintegration. The main objection that might be made to this method is that products from hydrolytic breakdown of muscle proteins by the action of heat and acid would be apt to give the color reaction for creatinine. In order to elucidate this point, extracts were obtained from frog muscle in the usual manner, from which proteins were precipitated by adding drop by drop a 5 per cent solution of acetic acid and gradually heating until the maximum precipitation was reached. The precipitated proteins were separated out by centrifuging, washed with water several times, and again separated by centrifugation. Portions of this precipitate were transferred to test-tubes, treated with 10 cc. of HCl of varying concentrations, and heated in the autoclave to

125° for 25 minutes. After cooling, the acid was neutralized
with NaOH with *p*-nitrophenol as indicator (2 drops of a 1 per cent
alcoholic solution) and the contents were transferred to a 25 cc.
volumetric flask and diluted to the mark with water. Then the
solution was transferred to another flask and 25 cc. of pure 1.2
per cent picric acid were added. Some precipitation occurred;

TABLE I.

Hydrolysis of Muscle Proteins.

Standard set at 20 mm.

Experiment No.	Concentration of HCl.	Average colorimetric reading.
	N	mm
1	0.2	20.3
2	0.2	19.1
3	0.2	20.4
4	1.0	19.5
5	5	20.5

TABLE II.

Addition of Solid Picric Acid to Protein-Free Filtrates.

Results are expressed in mg. of total creatinine per 100 gm. of tissue.

Tissue.	Controls without added picric acid	After addition of picric acid.
Beef muscle.	396	396
" "	408	400
" "	415	397
" "	402	395
" "	380	383
" "	382	397
Frog "	444	434
" "	396	400
" "	400	396

the fluid was now filtered and 25 cc. taken from the clear filtrate
to which 12.5 cc. of 5 per cent NaOH were added. After 10
minutes this solution was matched in the colorimeter against a
standard made by mixing equal volumes of HCl and NaOH of
the concentration used and adding 1 volume of 1.2 per cent picric
acid plus 1 of 5 per cent NaOH. In some cases the volume used
to make the standard was neutralized with NaOH with *p*-nitro-
phenol as indicator and the solution diluted to a certain volume.

The results are shown in Table I.

We use picric acid for the deproteinization of the hydrolyzed muscle extract. The objection could be made that, due to the precipitation of a portion of the picric acid by the protein materials, there would be a lower concentration of picric acid in the unknown solution than is contained in the standard.

Several control experiments were carried out to see whether this would lead to some appreciable inaccuracy in the results.

Series of estimations were made, a saturated solution of picric acid being used throughout. Every set of experiments consisted of four estimations on the same muscle. Two of them were

TABLE III.

Addition of Glycogen to Muscle.

Results are expressed in mg. per 100 gm. of tissue.

Frog gastrocnemius.	Glycogen added.	Creatinine found.
1		508
	194	490
2		490
	108	498
3		424
	191	422
4		414
	165	424

carried out in the usual manner as controls. To the protein-free filtrates obtained from the other two, solid picric acid was added in sufficient amount to provide for saturation. After mixing and standing for about 1 hour the fluids were filtered again and the four estimations carried on as usual. The results are summarized in Table II.

Most observers agree that the colorimetric comparisons should be completed 12 to 15 minutes from the time the color is developed. The basis for this statement is that carbohydrates also give the color test for creatinine, but the color due to these substances develops much more slowly than that due to creatinine alone. In several experiments we added glycogen to muscle in varying amounts and carried out creatine estimations by the

proposed method.   The figures so obtained were, within the limits of experimental error, not higher than those shown by control experiments when the color comparisons were completed 15 to 20 minutes from the time the NaOH was added to the unknowns; data illustrating this are summarized in Table III.

Pure picric acid as supplied by Merck was always used in estimations with the micro method, its purity for colorimetric work being checked by the test suggested by Folin and Doisy (2).

Solutions were often renewed to avoid error due to the develop-

TABLE IV.

Comparative Figures Obtained with Hahn and Schäfer's Method and with the Proposed Micro Method.

Beef muscle.   Results are expressed in mg. of total creatinine per 100 gm. of tissue.

Hahn and Schäfer.		Micro method.	
Weight.	Creatinine	Weight.	Creatinine.
gm.		*mg.*	
40	439	36	432
40	402	31	410
40	452	23	455
35	465	31	451
40	440	32	442
40	436	23	436
40	391	71	401
40	409	27	410
40	384	42	398
40	407	42	426

ment of chromogenic substances which takes place in old solutions of picric acid as pointed out by Hunter and Campbell (3).

It is also indispensable when one is working with concentrations of creatinine as small as those obtained in this method, to make out curves for the correction of the colorimetric readings as suggested by Hunter and Campbell (3); otherwise the results might be far from exact if the standard and the unknowns are not of nearly equal concentration.

Instead of these curves a correction factor might be introduced for each sample of picric acid used as Newcomb proposes (4).

We have used the proposed method in several hundred deter-

minations and have found it convenient and accurate. Experiments upon beef muscle have given comparable results with the method of Hahn and Schäfer (5), as shown in Table IV.

Analyses of muscle with and without the addition of known amounts of creatine and creatinine have yielded good recoveries; these data are summarized in Table V.

TABLE V.

Recovery of Creatine and Creatinine Added to Muscle.

Frog muscle. Results are expressed in mg. of total creatinine per 100 gm. of tissue.

Creatinine content.	Creatine added.	Creatinine added.	Creatinine found.	Recovered.
				per cent
329		200	528	99.8
531	143		690	102.3
400		52	433	96.2
374		114	471	96.5
531	86		588	95.4
410	142		532	96.3
409	112		510	97.8
378		200	590	102.0
363	221		588	100.6
368	235		602	99.8

TABLE VI.

Total Creatinine Content of Striated Muscle of Different Animals.

Results are expressed in mg. of total creatinine per 100 gm. of tissue.

Muscle.	No. of estimations.	Maximum and minimum values.	Average values.
Rabbit gastrocnemius..............	21	397–535	497
Frog "	20	460–533	487
Cat "	5	415–465	446
Frog sartorius....................	19	396–446	417
Beef...............................	22	380–467	414
Frog anterior rectus...............	14	327–434	382

In Table VI are shown the average figures calculated from determinations in different muscles of several animals.

Rose, Helmer, and Chanutin (6) have recently given a very similar method for the estimation of total creatinine in tissues

which provides for analyses on 1 gm. of tissue.   In experiments yet unpublished we have obtained comparable results with this method.

SUMMARY.

A method is given which allows the estimation of total creatinine in as small amounts of muscle as 5 to 100 mg.

The muscle creatine is extracted and converted into creatinine by HCl in the autoclave.   The proteins are precipitated by picric acid and the creatinine is colorimetrically estimated in the filtrate by the Folin technique.

The mean error as experimentally determined can be given as 0.5 to 2 per cent when all the operations are carried out with accuracy.

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REACTIVITY OF SOME CARBINOLS.

WITH A NOTE ON WALDEN INVERSION.

By P. A. LEVENE and ALEXANDRE ROTHEN.

(From the Laboratories of The Rockefeller Institute for Medical Research, New York.)

The number of investigations on the velocities of the interreaction of carbinols with halogen acids is very small.   More extensive is the work on the reactivity of organic halides and on the esterification of carbinols with organic acids.   However, even in the latter class of investigations, the types of carbinols and halides selected have not been sufficiently varied to permit comprehensive conclusions connecting reactivity with chemical structure.   The present investigation was undertaken, not with the intention of obtaining additional data, but rather with a specific purpose; namely, of finding an answer to the question as to whether or not there exists some connection between the reactivity of a carbinol and the occurrence or non-occurrence of Walden inversion in the course of its reaction with halogen acids. McKenzie[1] has made the observation that optically active methylphenyl carbinol on chlorination with phosphorus pentachloride yields a chloride rotating in the same direction as the carbinol; a halide rotating in the opposite direction is obtained when it has been acted upon by thionyl chloride.   In one of these reactions Walden inversion has undoubtedly occurred; in the other, it has not.   In the series of aliphatic alcohols halogenation with either reagent resulted in the identical halide.

In order to correlate the occurrence of Walden inversion with the molecular structure of the carbinols, Levene and Mikeska[2] have studied the result of halogenation by different reagents on a series of carbinols varying in their molecular structures.   The

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[1] McKenzie, A., and Clough, G. W., *J. Chem. Soc.*, **103**, 698 (1913).

[2] Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, **70**, 355 (1926).

variations to which they resorted are the following. (1) Aliphatic carbinols. (2) Methylcyclohexyl carbinol. (3) Benzylphenyl carbinol. (4) Mixed aliphatic aromatic carbinols. In this series the weight of the aliphatic radical was varied.

The graphic expression of the four types is presented in Formulæ I to IV.

I. II. III. IV.
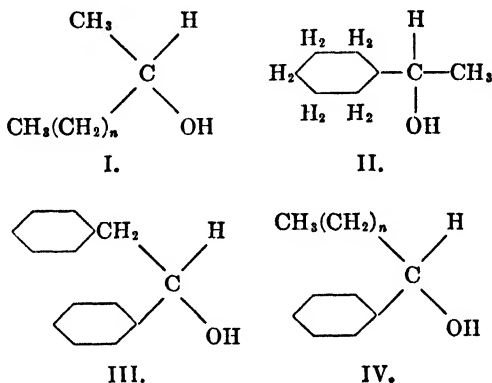
The observations of Levene and Mikeska led them to suggest that Walden inversion did not occur in the aliphatic series (1) and in benzylphenyl carbinol (3), and that it did occur in series (4) and in the methylcyclohexyl carbinol series (2). In each larger group there were observed variations of behavior of individual members. Thus methyl- and ethylphenyl carbinols formed with thionyl chloride halides rotating in the same direction as the carbinol, whereas with other reagents they formed halides rotating in opposite directions; on the other hand, in the higher homologues of this series (beginning with propylphenyl carbinol) an inversion seems to be produced by either reagent.

In articles on Walden inversion by Levene and Walti[3] and by Levene and Mikeska,[4] the idea was developed that the occurrence or non-occurrence of Walden inversion may be conditioned by the velocity of the reaction of substitution. Because of these considerations, it seemed desirable to compare the reaction velocities

[3] Levene, P. A., and Walti, A., *J. Biol. Chem.*, **73**, 263 (1927).

[4] Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, **75**, 587 (1927).

TABLE I.

Reaction of Alcohols with Hydrobromic Acid.

Temperature 79.7°, concentration 1.49 mols HBr, 100 mols alcohol, 12.8 mols water.

	Time required for:	
Normal primary alcohols.	25 per cent transformation.	50 per cent transformation.
	hrs.	*hrs.*
Amyl...	10.0	20
Hexyl..	8.5	17
Heptyl...	7.5	15 8
Octyl..	18.3	37
Primary isoalcohols.		
Isoamyl. $CH_3(C_2H_5)CHCH_2OH$	22	48
Isohexyl. $CH_3(C_3H_7)CHCH_2OH$	27	55
Isoheptyl. $CH_3(C_4H_9)CHCH_2OH$	50	76
Isooctyl. $CH_3(C_5H_{11})CHCH_2OH$	8	20
Benzylphenylethanol. $C_6H_5CH_2(C_6H_5)CHCH_2OH$	19.3	39.30
Secondary alcohols.		
Methylcyclohexyl carbinol.........................	86	114
Methylpropyl carbinol.............................	23.5	47
Diethyl carbinol.	10	26
Methylamyl carbinol..............................	14	31.5
Ethylbutyl "	12.5	26 5
Dipropyl carbinol.	15	32.5
Aromatic carbinols.		
Methylphenyl....................................	0.06	0.18
Propylphenyl....................................	0.06	0 16
Butylphenyl.....................................	0.1	0 22
Phenyl-*p*-tolyl. Benzylphenyl.	Same order of magnitude.	
Methylnaphthyl...................................	0.01	0 02

of the members of the series in which Walden inversion occurred with those in which it did not occur.   Inasmuch as the data on this subject recorded in the literature are limited to those given by Norris[5] in his lecture on "Chemical Reactivity," we undertook the investigation of the velocities of reaction in the carbinols of the types which served for observations on the Walden inversion. The results of our observations are presented in Table I.   From this table it is seen that a decisive and unmistakable difference in velocities of reaction is observed only between the aliphatic carbinols as one type and the mixed aliphatic aromatic carbinols as another type.   In the latter group the velocity of reaction is more than 100 times that of the aliphatic alcohols.

Differences of a second order are observed between different types of each class.   Thus in the aliphatic carbinols the slower rate of reaction is characteristic for the secondary carbinols and for the branched chain primary carbinols; the slowest rate is observed on methylcyclohexyl carbinol.

Thus, if methyl carbinol is taken as the parent substance of all carbinols and if for comparison four derivatives (VI, VIII, IX, X) all containing the same number of carbon atoms are taken, it is possible to formulate some idea as to the influence of substituting groups on the velocity of the reaction of halogenation by means of hydrogen halides.

$$
\begin{array}{cccc}
\text{H} & \text{H} & \text{CH}_3 & \text{H} \\
\text{CH}_3\text{—C—OH} & \text{C}_6\text{H}_{13}\text{CH}_2\text{—C—OH} & \diagdown & \text{H} \\
\text{H} & \text{H} & \text{CH(CH}_2)_3\text{CH}_2\text{C—OH} & \\
& & \diagup & \text{H} \\
& & \text{CH}_3 & \\
\text{V.} & \text{VI.} & & \text{VII.}
\end{array}
$$

$$
\begin{array}{cccc}
\text{H} & \text{H} & \text{H} & \text{H} \\
\text{CH}_3\text{—C—OH} & \text{CH}_3\text{—C—OH} & \text{CH}_3\text{—C—OH} & \text{CH}_3\text{CH}_2\text{—C—OH} \\
\text{C}_6\text{H}_{13} & & & \\
\text{VIII.} & \text{IX.} & \text{X.} & \text{XI.}
\end{array}
$$
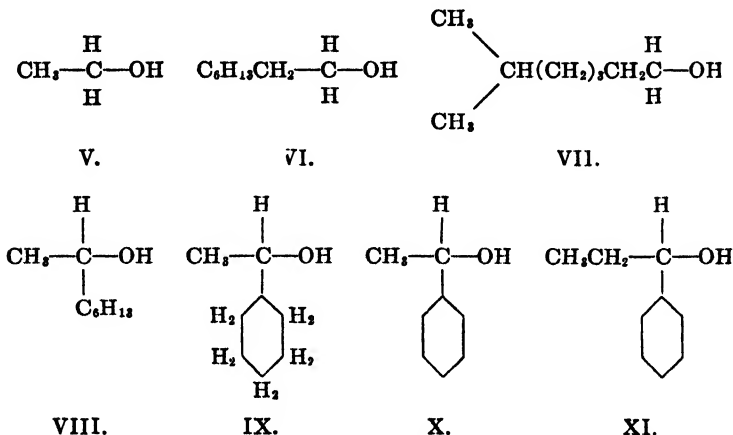
⁵ Norris, J. F., Contemporary developments in chemistry, New York, 1927.

If Formulæ VIII, IX, and X are compared, it is readily seen that the introduction of a polar group on the functional carbon atom produces the greatest effect on the reactivity of the carbinol. It seems as if the negative character of the phenyl group is transmitted to the significant carbon atom so that its power of attraction for the hydroxyl is lowered. On the other hand, its attraction for the hydrogen atom of the hydroxyl should be increased. And indeed, Norris and Cortese[6] have observed that the rate of esterification of the secondary . carbinols containing a phenyl group in position (1) is approximately a hundredfold as slow as the same reaction in the secondary aliphatic alcohols.

In the carbinols (VIII) and (IX) the substituting groups are of approximate'y the same polarity and of approximately the same mass and yet the effect of the substitution of these groups is not identical. The difference is of the second order of magnitude, but it is real. Thus it seems as if next to polarity the area or the distance of the center of gravity of the substituting groups determines the reactivity of the functional group. The influence of substitution of a branched chain for a normal chain in the primary carbinols is similar to that of substitution of a hydrocyclic radical for a normal radical in the secondary alcohols.

Thus it seems that the reactivity of carbinols is the result of several factors, each exerting a special effect on the degree of distortion of the molecule, or rather of the carbinolic carbon atom. The effect may be produced either by the polarity or by the mass, or by the area of the groups attached to the significant carbon atom.

The possibility still had to be considered that the reactivity of the carbinols is the result not so much of the molecular structure of the carbinols but of their physical properties. Of these the viscosity of the carbinols seemed of special importance. However, the measurements of the viscosities of the carbinols employed in our experiments have shown that they had no influence on the velocities of reaction (Table II).

Regarding the dynamics of the reaction, it must be said that the rate of reaction did not follow the simple monomolecular form of the mass law, as should be expected from the great excess of carbinol. However, calculating the velocity of the reaction for each interval as a monomolecular reaction and comparing the constants as a function of time, one may arrive at an expression for

[6] Norris, J. F., and Cortese, F., *J. Am. Chem. Soc.*, **49**, 2640 (1927).

the progress of the reaction of the character $K_t = K_0 + aC_t$ for the aliphatic, and $K_t = K_0 - aC_t$ for the mixed aliphatic aromatic carbinols.

However, for the sake of simplicity, the velocities of reaction were compared by measuring the time of 25 and of 50 per cent of halogenation (Table I).

The bearing of these results on the mechanism of the Walden inversion will be discussed in detail later. Here it may be said that at first glance it seems that the observations are contrary to

TABLE II.

Viscosities of Alcohols at 25° ±0.01°.

Normal primary alcohols.		Secondary alcohols.	
Amyl.	0.0323	Methylcyclohexyl carbinol.	0 264
Hexyl.	0.0435	Methylpropyl carbinol.	0 0321
Heptyl.	0.0477	Methylamyl carbinol.	0 0228
Octyl.	0.0520	Ethylbutyl "	0 0213
		Diethyl carbinol.	0 0163
Primary isoalcohols		Dipropyl "	0 0163
Isoamyl. $CH_3(C_3H_5)CHCH_2OH$	} 0 0386	Aromatic carbinols	
Isohexyl. $CH_3(C_3H_7)CHCH_2OH$	} 0 0362	Methylphenyl.	0 0849
		Propylphenyl.	0.114
Isoheptyl. $CH_3(C_4H_9)CHCH_2OH$	} 0 0553	Butylphenyl.	0.173
Isooctyl. $CH_3(C_5H_{11})CHCH_2OH$	} 0 0424		

the expectation, inasmuch as Walden inversion occurs principally in the mixed aliphatic aromatic series in which the velocity of substitution is the greatest. In the discussion of the connection between velocity of reaction and the Walden inversion, not the absolute velocity was emphasized, but the differences between the velocity of substitution and that of transposition of two groups within the molecule. It is quite possible that in the mixed aliphatic aromatic series the mobilities of all the groups are very high and that the reactions within the molecule take place at a still greater velocity than the reaction of substitution. Indeed in methyl- and ethylphenyl carbinols, in which only one reagent

brings about Walden inversion, Levene and Mikeska were led to believe that thionyl chloride was the reagent bringing about Walden inversion. In this case the thionyl chloride reaction is the slower one, inasmuch as the initial step in it is the formation of

an ester of the type $H—\overset{\displaystyle R'}{\underset{\displaystyle R}{\overset{|}{\underset{|}{C}}}}—O—SOCl$. The work of Norris and

Cortese[6] has already been referred to, in which it was demonstrated that the substitution of the hydrogen atom of the hydroxyl in the mixed aliphatic aromatic carbinols proceeds at a low rate.

The case of benzylphenyl carbinol deserves special consideration. In this carbinol the velocity of reaction is of the same order of magnitude as in the mixed aliphatic aromatic carbinols and yet Levene and Mikeska have reached the conclusion that the halogenation of this carbinol proceeds without Walden inversion. This exceptional behavior may be explained on the assumption that the similarity of the benzyl and phenyl radicals brings about a greater symmetry of the molecule on one hand, and on the other, that the negative character of both groups may increase the distance between them because of their mutual repulsion.

In the aliphatic series, Walden inversion thus far has been observed in one instance only, namely in methylcyclohexyl carbinol, and this was found to be the least reactive of all secondary carbinols.

<div align="center">

EXPERIMENTAL.

Materials.

</div>

Preparation of Hydrobromic Acid.—Hydrogen bromide was prepared according to Murray;[7] *i.e.*, by allowing 100 per cent phosphoric acid to drip on crystallized potassium bromide. A very regular evolution of fairly pure hydrogen bromide was thus obtained. The latter was liquefied and distilled three times before it was used to fill a 5 liter reservoir.

[7] Murray, W. J., *J. chim. physique*, **15**, 344 (1917).

This preparation was made in a sealed glass apparatus of the type often used and described for the physical purification of gases.

The liquefaction of hydrogen bromide was achieved by a mixture of solid CO_2 and alcohol.

Carbinols.—The carbinols were carefully purified and distilled and used only after giving a correct analysis.

General Procedure.

To obtain comparable results, all the experiments with the different alcohols were made at the same molecular concentration. The apparatus was so built that the solution of a known volume of hydrogen bromide at a known temperature and pressure in a weighed amount of alcohol was easily brought about. By modifying the pressure and by using three different calibrated volumes (6.315 cc., 22.53 cc., 242 cc.) absolute amounts of HBr varying from 0.003 gm. to 1 gm. could be dissolved in the alcohols and measured with a precision of at least 1 per cent. The small amount of HBr used for each run was always liquefied and distilled before the run.

As has already been observed by Norris, the water formed as the reaction proceeds, $ROH + HBr \rightarrow RBr + H_2O$, decreases the rate of the reaction. Even with such a low concentration as $\dfrac{1\ HBr}{1000\ alcohol}$ the water formed greatly affects the velocity of the reaction.

Two series of experiments were made: one at high concentration of HBr $\dfrac{15\ HBr}{100\ alcohol}$ and the other at low concentration $\dfrac{1.49\ HBr}{100\ alcohol}$. In the second series an initial amount of 12.8 mols of H_2O was present.

Temperature of Reaction.—The reactions were carried out at three different temperatures: 79.7°, 55°, and 25°.

As soon as the mixture had been made, it was placed in a closed vessel at the desired temperature obtained by an oil-filled thermostat; a water-filled thermostat was used for 25°. The thermostat was heated electrically by a coil of nichrome wire. The thermoregulator device was of the type described by Beaver and Beaver,[8]

[8] Beaver, D. J., and Beaver, J. J., *Ind. and Eng. Chem.*, **15**, 360 (1923).

with a radio tube in the breaking circuit.   The temperature was kept constant ($\pm0.05°$) for weeks.

Method of Analysis.—The reaction was followed by measuring from time to time the amount of hydrogen bromide remaining. In order to economize the alcohol, small amounts were used and each titration was made with 0.2 to 0.3 gm.   Two methods were used.   In each of them a certain amount of water (4 cc.) was added to the sample to be analyzed.   After agitation and centrifugalization the water layer contained all the free HBr, as shown by some blank runs.   In the first method the HBr in solution was titrated by conductivity titration with 0.01 N $AgNO_3$ as reagent.

The sensitiveness was such as to permit the titration of 0.00001 mol with a precision of 0.5 per cent.   As is known, the end-point of the titration is given by a sharp angle in the value of the curve obtained by plotting conductivity against the amount of reagent introduced.   For the conductivity titrations a small cell of 10 cc. was used.   When working at higher concentrations the second, quicker, method was used.

The HBr water extract was placed in a special cell and water added to a definite volume.   The conductivity of the solution gives directly the amount of HBr, the conductivities of HBr solution at different concentrations having been determined previously. For the dilutions used (0.003 to 0.1 per cent) the curve of conductivity against concentration is practically a straight line. The precision is of 0.1 per cent.   Some blank tests have shown that hydrolysis of the bromide as well as the change of conductivity due to the different solubilities of alcohol in water was negligible for most of the alcohols studied.

Conductivity Measurements.—The conductivity measurements were made with a Wheatstone bridge of the type recommended to us by Dr. MacInnes.   The ratio of the arms $\frac{a}{b}$ was equal to $\frac{1000\Omega}{1000\Omega}$ = 1 $\pm$ 0.0001 thus allowing a direct reading of the resistance. The bridge was earthed according to Wagner.   It was supplied by an alternating current furnished by an oscillating circuit (radio tube, convenient capacity and inductive coils).   The measurements were made with an oscillating frequency of 1000 per second.

The bridge was calibrated according to Parker and Parker.[9]

Resistances up to 50,000 ohms and, by changing the ratio of the arms to $\frac{1}{10}$, up to 500,000 ohms, could be measured. Each reading was accurate to 0.01 per cent.

The electrodes of the cells were either slightly platinized or not platinized at all. Each conductivity measurement was made at 25°. The cells were placed in a large thermostat, the temperature of which was kept constant within ± 0.003°. The thermoregulator was of the same type as that described previously.

Results.

The results obtained are given in Table I.

The time values given in Table I are those obtained for the esterification at 79.7°. The reaction with the aromatic carbinols was carried out at 55° and the velocities for 79.7° were calculated from the temperature coefficient of the reaction.

The solid carbinols were studied in ether solution at 25°. The effect of the solvent was easily determined by checking the results with a liquid carbinol the esterification of which was studied both with and without ether.

Consistent results for phenyl-*p*-tolyl carbinol and benzyl-phenyl carbinol could not be obtained because of the instability of the bromide compound in water.

[9] Parker, H. C., and Parker, E. W., *J. Am. Chem. Soc.*, **46**, 316 (1921).

THE ISOLATION OF PROTOCATECHUIC ACID FROM PIGMENTED ONION SCALES AND ITS SIGNIFICANCE IN RELATION TO DISEASE RESISTANCE IN ONIONS.*

By KARL PAUL LINK,

(From the Department of Agricultural Chemistry, University of Wisconsin, Madison.)

AND H. R. ANGELL AND J. C. WALKER.

(From the Department of Plant Pathology, University of Wisconsin, and the Office of Horticultural Crops and Diseases, Bureau of Plant Industry, United States Department of Agriculture Field Laboratory, Madison.)

INTRODUCTION.

In a series of investigations published by one of us (Walker, 1–4) it was shown that the red and yellow varieties of the common bulb onion (*Allium cepa*) are in general resistant to the disease caused by the fungus, *Colletotrichum circinans* (Berk) Vogl., whereas the white varieties are susceptible. The investigations revealed that an aqueous extract of the dry outer pigmented scales causes rupturing or abnormal germination of the spores and retards the growth of the mycelium of *Colletotrichum circinans*, whereas a similar extract from the dry outer white scales is not endowed with this property.

It was stated guardedly and with the necessary reservations ((2) p. 1036) that the chief factor imparting the resistant property to the pigmented onion scales was a substance or group of substances either closely associated or identical with the red and yellow pigments present.

In this publication we report the isolation and identification

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of *one toxic entity* obtained from the pigmented onion scales which is not present in the white scales. The toxic entity has been definitely characterized and identified to be the phenolic acid commonly known as protocatechuic acid (3, 4-dihydroxybenzoic acid).

Occurrence of Protocatechuic Acid in the Plant Kingdom.

Although protocatechuic acid is widely distributed in plants as a constituent of many aromatic compounds, in the catechol tannins, in numerous resins and wood gums, in lignified wood, and as a constituent of various flavone and anthocyan pigments ((5) p. 1297), ((6) p. 479), ((7), p. 641), its occurrence in the free state has been reported only in a few cases.

Perkin (8) reported its occurrence in the flowers of *Hibiscus sabdariffa*, a red sorrel of the West Indies, and in the flowers of *Thespesia lampas*, a small bush common to tropical jungles of India, Burma, and Ceylon. Free protocatechuic acid has also been reported present in the fruits of *Illicium anisatum*, the Chinese anise, by Eykman ((9) p. 214) and in the leaves of the wine grape, *Vitis vinifera*, by Boettinger ((9) p. 213).

It is of sufficient importance to make note here that the flavonol quercetin was also present in the two plants from which Perkin (8) isolated protocatechuic acid. The isolation of protocatechuic acid from pigmented onion scales thus represents the third instance in which the acid has been found associated with the flavonol quercetin. Perkin and Hummel (10) had also reported the isolation of quercetin from pigmented onion scales.

Upon alkaline fusion the pigment quercetin breaks up into phloroglucinol, oxalic acid, and protocatechuic acid.

Significance of the Isolation of Protocatechuic Acid from Pigmented Onion Scales.

We wish to state here that the toxic action of the pure protocatechuic acid isolated, in dilutions of 1 part to 3000 parts of water, is identical with the toxic activity of the crude active aqueous extract from which it can be isolated. The toxicity of the crude aqueous extracts is, however, greater than the toxic effects that could be ascribed to the amount of protocatechuic acid that we have isolated from a given unit of toxic extract.

From the aqueous extract of 100 gm. of the dry pigmented scales we were able to isolate approximately 0.1 gm. of the pure acid.

This leads us to the belief that the quantity of protocatechuic acid isolated either represents only a fraction of the total present or suggests the alternate contingency that there are present still other phenolic substances or groups of substances to which some of the toxicity of the aqueous extract can be ascribed.

We are therefore extending the investigations with the purpose of isolating other toxic substances from the aqueous extract of pigmented scales and if possible to increase the yield of the protocatechuic acid.

As far as we are able to ascertain from the literature on disease resistance in plants, this is the first instance wherein resistant properties can be ascribed to a chemical entity present in resistant varieties (the pigmented onions) and absent in the non-resistant varieties (the white onions).

Preliminary Experimentation for Toxic Principles.

Various clues obtained in the progress of the experimentation[1] led us to believe that the toxic entities present in the pigmented onion scales were either closely associated with derivatives of phenolic substances, or phenolic substances existing in the free state. Perkin and Hummel (10) had reported the occurrence of quercetin, the 1:3:3'-tetrahydroflavonol in pigmented onion scales. The quercetin was isolated by boiling the onion scales repeatedly with hot water. Quercetin is, however, markedly insoluble in cold water. Since it was shown that the toxic entities that we were working with were readily diffusible in cold water, it seemed unlikely that the toxicity of a cold, aqueous extract could be due to quercetin. We should make note here that one of us had removed the quercetin present in an acetone extract obtained from the pigmented scales, which also contained the water-soluble entities. The extract (quercetin-free) was still toxic to the fungus *Colletotrichum circinans*. The details of this work will be described elsewhere.[1]

It was noted that the cold aqueous extract from the pigmented

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[1] Consult the paper, "A Chemical Compound Responsible for Resistance in the Onion," in the *Journal of Agricultural Research*, in press.

onion scales turned pink in acid solutions and brown in alkaline solutions. These color changes are characteristic of the anthocyan and flavone pigments. Hydrolytic experiments conducted in 2.0 per cent aqueous sulfuric acid and 4.0 per cent sulfuric acid containing 45.0 per cent ethyl alcohol for 6 hours according to the procedure of Van der Haar ((11) pp. 304–305) showed that there was no substance present in a glucosidic combination in the aqueous extract. The quantity of sugar isolated (glucose) before hydrolysis approximated that obtained after acid hydrolysis. These trials substantiate the work of Perkin and Everest ((12) pp. 201–202) who state that attempts to isolate a quercetin glucoside or other glucosides from onion scales had failed. We are inclined to the belief that any member of the flavone or anthocyan group existing in the cold aqueous extract obtainable from the pigmented onion scales must exist in the free state; that is, as the non-glucosidic or anthocyanidin form.

Although it is reported by Dekker ((13) p. 118) and Wehmer ((9) pp. 95–96) that no true tannin-bearing substances have been isolated from the members of the Lileaceæ family, to which the onion belongs, we did not overlook the possibility that a tannin-like substance might be present in the toxic extracts. When the residue from the aqueous extraction was subjected to the prolonged hydrolytic procedure recommended by Fischer and Freudenberg for tannin hydrolysis (14) (60 to 70 hours in 6.0 per cent aqueous sulfuric acid), the quantity of sugar that could be isolated did not exceed the amount obtained before the hydrolysis.

The toxicity control experiments conducted along with the chemical investigations indicated that the toxic principles belong to the class of substances that give a color reaction with a solution of ferric chloride. The color produced with ferric chloride in neutral or slightly acid solution is at first a grass-green, which soon changes to a greenish purple. Upon standing, an amorphous blue-green precipitate settles out. This is a reaction characteristic of catechol derivatives. We were thus led to search for phenolic substances existing in the free state.

The protocatechuic acid was isolated after the following procedure.

Method of Isolation.

The dry pigmented onion scales were digested with 20 times their weight of water at 30° for 2 hours and then pressed free from the liquors in a hand press. The extraction was repeated with a fresh quantity of water. The quantity of quercetin thus removed is practically nil. To the combined extraction liquors, basic lead acetate was added until complete precipitation had been effected. An excess of the acetate was avoided. The voluminous reddish brown lead precipitate obtained was filtered off at the pump and washed with cold water. •It was then suspended in a large volume of water by shaking upon a machine and decomposed with hydrogen sulfide. The lead sulfide was removed and the filtrate freed from hydrogen sulfide by aspiration. The solution was then concentrated under reduced pressure at 40–50° until the syrup contained 80 to 85 per cent of solids. Upon standing for 12 hours, a dark brown gum separated, leaving a thinner aqueous syrup which gives a strong positive test with a solution of ferric chloride. From this syrup most of the toxic principles could be removed by adding successive portions of cold 95 per cent ethyl alcohol and quickly removing the alcohol liquors before the gummy precipitate went into solution.

The alcoholic extracts thus obtained are strongly pigmented, usually a deep red. This pigmentation, we believe, is due to either a member of the anthocyanidin or the flavone group. Most of the pigment can be removed by heating the alcoholic extract under a reflux condenser in the presence of successive small portions of activated blood charcoal. The charcoal used in this work was rendered active by heating in a covered nickel crucible on a sand bath until it ceased to give off water vapor and air. It was then allowed to cool in a desiccator over calcium chloride. After three treatments with the activated charcoal the alcoholic solution attained a light brown color, but still gave a very positive color reaction with a solution of ferric chloride, indicating that the toxic phenolic substance or substances were still intact.

The alcoholic solution was concentrated under reduced pressure at 35°. The yellow syrup obtained was taken up in warm 20 per cent alcohol, and warm petroleum ether, b.p. 60–80° (1 part to 5), was added. Upon standing in a desiccator over calcium chloride, crystals collected upon the sides of the crystallizing vessel. These

crystals, after drying in a vacuum of 15 mm. at 90° over P_2O_5, melted at 195°.   Upon further purification, with water as the solvent, the crystals were obtained as monoclinic needles with a constant m.p. of 199°.   These crystals were found to be toxic to the organism causing the disease.   For the detailed report upon the toxicity tests conducted, consult the paper to be published by us in the *Journal of Agricultural Research.*

The crystalline substance obtained as described above was identified and found to be the dihydroxyphenolic acid known as protocatechuic acid.   The acid was obtained in yields approximating 0.1 gm. per 100 gm. of the dry scales.

Identification of the Protocatechuic Acid.

A *mixed melting point determination* made with an authentic specimen of protocatechuic acid obtained by fusing East Indian kino with potassium hydroxide after the method of Stenhause (15), gave no depression of m.p. 199°.   The mixed melting point determination was also controlled with a specimen of the acid obtained from the Kahlbaum chemical works.

The *methyl ester* $C_8H_8O_4 = 3$, 4-dihydroxybenzoic acid methyl ester prepared by condensing the isolated protocatechuic acid with dry methyl alcohol containing 1 per cent of hydrogen chloride after the method of Meyer (16), showed a m.p. of 134.5°, which is identical with the constant reported by Meyer.

The *ethyl ester* $C_9H_{10}O_4 = 3$, 4-dihydroxybenzoic acid ethyl ester prepared by condensing the isolated protocatechuic acid with dry ethyl alcohol containing 1 per cent hydrogen chloride, after the method of Fittig (17), showed a m.p. of 134°, which is identical with the constant reported by Fittig.

The *diacetyl derivative* $C_{11}H_{10}O_6 = 3$, 4-dihydroxybenzoic acid diacetyl derivative, prepared by boiling the isolated protocatechuic acid with acetic anhydride in the presence of zinc chloride, after the method of Herzig (18), gave a m.p. of 157–158°, identical with the m.p. reported by Herzig.

The ultimate analysis gave C 54.50 per cent,[H 3.92 per cent (Pregl micro method).[2]   Theory demands for $C_7H_6O_4$, C 54.52 per cent, H 3.94 per cent.

SUMMARY.

Protocatechuic acid (3, 4-dihydroxybenzoic acid) has been isolated from pigmented onion scales. This phenolic acid appears to be one of a group of toxic substances that enables the pigmented onions to resist the inroads of the fungus, *Colletotrichum circinans*, the organism responsible for the disease commr 'v known as onion smudge.

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THE NATURE OF BLOOD SUGAR.

By OTTO FOLIN.

(From the Biochemical Laboratory of Harvard Medical School, Boston.)

In the December number of this *Journal,* Somogyi and Kramer (1) have a paper under the above title in which they prove to their own satisfaction that there is no other sugar in blood than glucose and that the contrary findings of Folin and Svedberg (2) must be ascribed to the use of faulty methods.

It must be freely admitted that the new copper method used in the work of Folin and Svedberg may not be sufficiently dependable to warrant the acceptance of so important a conclusion as the presence of another sugar than glucose in blood. The work certainly needs confirmation. But the results reported by Somogyi and Kramer have little if any bearing on the subject, and their conclusion that the only sugar in blood is glucose is based on unwarranted premises.

The characteristic feature of Folin's new copper method as used by Folin and Svedberg is that it gives much lower blood sugar values than the method of Folin and Wu, yet gives, or seemed to give, dependable values for glucose. The "non-glucose sugar," whose presence in blood was postulated by Folin and Svedberg, is a sugar with so much lower reducing power than glucose that while it might be completely included in the Folin-Wu determinations, it escaped determination, in whole or in part, when the more weakly alkaline copper reagent of Folin was used.

While Somogyi and Kramer used three different methods for the determination of blood sugar, their lowest blood sugar values (before fermentation) were obtained by the method of Folin and Wu. The two titration methods used gave much higher values; indeed, one, the ferricyanide method, gave extraordinarily high values. These methods as used by Somogyi and Kramer determined, therefore, not only everything in the way of sugar, but a

great deal besides sugar. There could be no possibility, of course, of finding the hypothetical non-glucose sugar of Folin and Svedberg by the use of such all inclusive methods.

There are some technical points in the paper of Somogyi and Kramer upon which it seems suitable to make a few comments.

Somogyi is rather free in the suggestion and use of modifications, so much so that one cannot always be quite sure just how a method is employed by him. He now suggests that the copper reagent in the method of Folin and Wu should have the following composition:

	gm.
Sodium carbonate	20
" bicarbonate	25
Copper sulfate	7
Rochelle salt	25

The advantage gained is a deeper color which is desirable when working with very weak reducing liquids such as fermented blood filtrates. It is particularly pointed out that Rochelle salt instead of tartaric acid should be used because with the latter one loses more or less CO_2.

As it happens, the reagent thus proposed is a particularly unsuitable one for the determination of the sugar in very dilute solutions.

The reason why the Folin-Wu copper reagent has remained so satisfactory is that it was about the first copper tartrate reagent which would keep indefinitely, and gives very little autoreduction or blank when heated with water. This essential characteristic is obtained partly by using tartaric acid instead of Rochelle salt for holding the copper in solution, but above all by keeping the copper sulfate and tartrate concentration as low as possible. By increasing the copper sulfate content from 4.5 gm. to 7 gm., and replacing 7.5 gm. of tartaric acid with 25 gm. of Rochelle salt one gets a reagent which is much inferior, if not actually useless, for accurate work with very dilute sugar solutions, because of autoreductions in blank experiments. So far as can be seen from their paper, Somogyi and Kramer have made no tests for the color in blank experiments (a precaution of fundamental importance in colorimetry).

Somogyi and Kramer have also come to the conclusion that the use of the Folin-Wu sugar tube represents an unnecessary refinement, and cite figures to show that the reoxidation of cuprous oxide in open test-tubes is so small as to be negligible. Curiously enough, they obtained no loss at all by such reoxidation in the most important experiment with 0.002 per cent of sugar, which corresponds approximately to the reduction of fermented blood filtrates. According to my experience the losses in test-tubes with an internal diameter of 15 to 16 mm. amount to not less than 25 per cent when one is working with 0.002 per cent glucose solutions.

Somoygi seems to have worked more or less exclusively with titration methods for the determination of sugar, and he emphasizes the fact that in his hands and Kramer's, the colorimetric method gives rather inconsistent results. It may be fairly suggested, I think, that this is not the fault of the method. As a matter of fact, the ferricyanide method also has not given entirely reasonable results in the hands of Somogyi and Kramer. The figures reported are certainly not the kind of figures which should be obtained by the Hagedorn-Jensen method.

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AN ADAPTATION OF BERGEIM'S FECAL INDOLE METHOD FOR THE QUANTITATIVE DETERMINATION OF INDOLE IN BACTERIAL CULTURES.

By H. B. PIERCE and R. B. KILBORN.*

(*From the Department of Vital Economics, and the Department of Bacteriology, School of Medicine and Dentistry, The University of Rochester, Rochester, New York, and Department of Agricultural and Biological Chemistry, Pennsylvania State College, State College.*)

During the course of a recent investigation, one of the writers (P.) desired to determine quantitatively the amount of indole present in bacterial cultures. Prior to this time, Bergeim's method (1) had been found to be satisfactory for the estimation of indole in feces.

Bergeim believed that of the large number of color reactions which had been used for the determination of indole, only the β-naphthoquinone sodium monosulfonate reaction of Herter and Foster (2, 3) offered much promise as a basis for the accurate determination of indole in feces. On the basis of this experience we have investigated the applicability of this method for the quantitative determination of indole in bacterial cultures.

Bergeim's method consists of a steam distillation of a feces suspension in an alkaline medium to remove phenols. The distillate, which contains indole and ammonia, is redistilled from an acid solution or treated with permutit (exchange silicate) to remove ammonia. A portion of the ammonia-free distillate is treated with β-naphthoquinone sodium monosulfonate, the blue indole compound formed being extracted with chloroform and determined colorimetrically.

A survey was made of other methods which had been recom-

* Mr. Kilborn assisted in the later studies made at Pennsylvania State College.

mended for the determination of indole quantitatively in bacterial cultures.   Fellers and Clough (4) made a comprehensive study of this problem several years ago, and discussed the relative merits of the various methods which had been used prior to the time their work was undertaken.   They have classed the Herter and Foster method as "fairly satisfactory" but they do not recommend Bergeim's modification of this procedure for accurate work. They concede that the method is useful for separating indole quantitatively from skatole but they say that "the procedure is involved and the reagent exceedingly difficult to obtain."   Another objection was that the reagent did not keep well on standing. After studying a number of procedures, Fellers and Clough decided to use the Ehrlich (5) p-dimethylaminobenzaldehyde reaction for their determination of indole.

Zoller (6) has objected to the use of Bergeim's method because (a) indole had to be separated from phenols and ammonia and this procedure was involved, (b) the reagent deteriorated rapidly and was difficult to obtain, and (c) a colorimeter had to be available for the determination.

The reagent may be obtained from the Eastman Kodak Company at present, the price of 10 gm. being 90 cents.   This amount of β-naphthoquinone sodium monosulfonate is sufficient for approximately 500 determinations, and, if the solution containing this reagent is kept in the ice box, it does not deteriorate during a period of 3 days.   When a precipitate forms, the reagent should be discarded.   The separation of indole from phenols requires distillation from an alkaline medium and the subsequent removal of ammonia by the use of permutit or a second distillation from an acid solution.   This procedure does require time, but it is not at all difficult to carry out with average equipment, and a colorimeter is usually available in modern laboratories.

EXPERIMENTAL.

Before Bergeim's method was used to determine the amount of indole present in bacterial cultures, a study was made of the percentage of indole recovered from water solutions, sterile 2 per cent solutions of Witte's peptone in water, and bacterial cultures in peptone water, to which definite amounts of a standard indole solution had been added just prior to the distillation.   The

bacterial cultures were prepared by inoculating 100 cc. of peptone water with 0.2 cc. of a feces suspension prepared according to Torrey (7), the indole being determined after 48 and 96 hours incubation periods. A definite amount of the culture medium, usually 10 cc., was removed and diluted to 400 cc. before distilling. The water solutions of indole were prepared by adding known volumes of a standard indole solution to 400 cc. of water, while the peptone water solutions were prepared by diluting definite amounts of peptone water, usually 10 cc., to 400 cc. with water and adding known volumes of a standard indole solution. The solutions containing indole were distilled from 800 cc. Kjeldahl flasks which were attached to connecting bulbs leading to 24 inch Liebig condensers arranged in an upright position. Glass delivery tubes were fitted to the ends of the condensers so that the distillate passed directly into 50 cc. of water in the receivers which were 500 cc. volumetric flasks. Prior to the distillation process, 5 cc. of 10 per cent KOH and approximately 2 gm. of paraffin were added, the latter to prevent foaming. The amount of KOH added placed the pH in the range of 8.5 to 10.0, which Zoller (8) found to be optimum for the recovery of indole from solutions. The distillation was carried out by the combined use of a flame and steam. The steam, obtained from a high pressure line (about 50 pounds) was passed through the contents of the flask until the volume in the receiving container amounted to 500 cc. This insured a maximum recovery of the indole which had been added to the solutions.

The initial heating must be carried on with caution to avoid excessive foaming and loss of indole by a too rapid volatilization. Although the larger amount of indole passes over with the first 100 cc. of the distillate, as Fellers and Clough have pointed out, it has been observed by the writers that some indole remains unvolatilized and the method loses its quantitative aspect unless approximately 500 cc. of the distillate are collected and the volume of the contents of the Kjeldahl flask is reduced to about 150 cc. Water is added to the solution by the condensation of steam, and if the flow of steam is too rapid, it is impossible to reduce the final volume to 150 cc. The steam flow may be controlled by regulating outlet valves on the steam line.

50 or 100 cc. of the distillate, depending upon the amount

of indole present, were transferred to a 500 cc. Erlenmeyer flask and 20 gm. of washed permutit[1] were added. The flask and contents were rotated moderately for 10 minutes, then, as soon as the permutit settled, the liquid was poured into a 150 cc. pear-shaped separatory funnel. The flask was rinsed with 5 to 10 cc. of water twice, the rinse water being decanted and added to the contents of the separatory funnel. 1 cc. of a 2 per cent solution of β-naphthoquinone sodium monosulfonate and 2 cc. of 10 per cent KOH were added, the contents of the funnel shaken, and the mixture allowed to stand for 15 minutes. A greenish-blue color developed and this colored compound was extracted first

TABLE I.

Indole Recovery from Water Solutions, Peptone Water, and Bacterial Cultures in Peptone Water.

Series No	Medium.	Indole added	Average indole recovered	No of determinations
		mg.	*per cent*	
1	Water solutions	0 2–2 0	94 62	40
2	" " distilled with steam.	5 0	96 92	34
3	Water solutions distilled without steam	5 0	88 58	18
4	2 per cent Witte's peptone water.	0 5–10 0	90 82	33
5	Bacterial cultures in 2 per cent Witte's peptone water.	0 5–5 0	90 85	28

with a 10 cc. portion of chloroform and again with a 7 cc. portion. The chloroform extract was drawn off each time into a 15 cc. graduated cylinder The amount of solvent used was sufficient to yield a final volume of 15 cc. The standard was prepared similarly and at the same time as the unknown by using 1 cc. of an indole solution containing 0.1 or 0.2 mg., diluted to 100 cc. The standard was usually set at 30 mm. in the colorimeter.

Table I shows the results obtained during a study of the recovery of various amounts of indole from water solutions,

[1] The permutit is washed to remove very fine particles which would otherwise cause a cloudiness in the solution to be used for the colorimetric comparison. The permutit may be recovered.

peptone water, and bacterial cultures in peptone water to which definite amounts of indole had been added after incubation.

The average percentage of indole recovered from water solutions of indole in Series 1 was 94.62 per cent. The results in Series 2 and 3 illustrate the importance of using steam throughout the distillation period. The percentage of indole recovered by using steam continuously during the distillation was 96.92, while that recovered without the use of steam was 88.58. This result was to be expected, for substances relatively insoluble in water and possessing an appreciable vapor pressure àt 100° are removed more

TABLE II.
Indole Recovered from Bacterial Cultures.

Indole in culture.	Indole added.	Indole found.	Indole recovered.
mg.	*mg.*	*mg.*	*per cent*
10.53	0.5	11.05	104.0
7.61	0.5	8.03	84.0
10 05	1.0	10.94	89.0
11.80	1.0	12.71	91.0
2.99	1.0	3.95	96.0
1.66	2.0	3.51	92.5
9.30	2.0	11.06	88.0
10.58	3.0	13.29	90.3
10.98	3.0	13.75	92.3
10.84	3.0	13.49	85.0
8.06	5.0	12.65	91.8
12.42	5.0	16.99	91.4
13.20	5.0	17.81	92.2
14.34	5.0	18.97	92.6

efficiently from solution by steam distillation. The determinations made in Series 4 and 5 show the percentage of added indole recovered from sterile peptone water and bacterial cultures in peptone water. Approximately 91 per cent of the indole which had been added was recovered. Table II shows in detail the recovery of indole from bacterial cultures to which known amounts of indole had been added just prior to distillation. There appears to be a retention of indole by the peptone water. Fellers and Clough have observed this same phenomenon in the case of experiments in which attempts were made to remove indole from decomposing protein matter.

Attention has been called to the interference of the pale yellow color of the naphthoquinone reagent which modifies the pink color of the chloroform extract, but according to Bergeim this is scarcely appreciable when as much as 0.1 mg. of indole is present. With smaller quantities of indole this factor cannot be neglected. The standard used should contain approximately the same amount of indole as the unknown. Chloroform tends to form an emulsion during the extraction of the colored indole compound. The emulsion breaks within 1 or 2 minutes. It was observed that chloroform containing a trace of water did not tend to emulsify as readily as chloroform which contained no water.

The standard indole solutions should be freshly prepared, although, if kept in the ice box, little or no change takes place during periods of 3 or 4 days.

The color fades but slowly from the chloroform extracts, there being no appreciable change during a 20 minute period following the extraction.

Permutit (exchange silicate) proved to be entirely satisfactory for the removal of ammonia from the distillate.

Bergeim found indole to be removed quantitatively from solution by his process of distillation. We have found that an average of 90 per cent of the indole is removed from bacterial cultures under the conditions enumerated. Although the method is not 100 per cent quantitative, it is more nearly so than other methods which have been devised.

SUMMARY.

1. Bergeim's method for the determination of fecal indole has been adapted to the quantitative estimation of indole in bacterial cultures.

2. An average of 91 per cent of the indole added to peptone water or to bacterial cultures in peptone water has been recovered by use of this method.

3. The average percentage of indole recovered from water solutions ranged between 94 and 97.

4. Steam should be passed through the contents of the distilling flask during the entire period of distillation and the solution remaining in the flask at the end of the period should amount to approximately 150 cc.

5. The method outlined in the preceding discussion is more nearly quantitative than the method of Fellers and Clough, and the procedure, once the technique is standardized, is not involved.

6. The reagents required are comparatively inexpensive and may be obtained without difficulty.

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GLYCOGEN FORMATION IN THE LIVER FROM d- AND l-LACTIC ACID.

By CARL F. CORI and GERTY T. CORI.

(From the State Institute for the Study of Malignant Disease, Buffalo.)

The question as to whether or not the liver is able to form glycogen from lactic acid is of importance for the relation between the carbohydrate metabolism of the muscles and that of the liver. It is known that the process glycogen $\rightleftarrows$ lactic acid occurs in the muscles, while the process glycogen $\rightleftarrows$ glucose occurs in the liver. Glucose derived from liver glycogen is convertible into muscle glycogen; it is, however, not definitely known whether lactic acid derived from muscle glycogen is convertible into liver glycogen. If this should prove to be the case, the glucose molecule would be capable of a complete cycle in the body; it could in turn be liver glycogen, blood sugar, muscle glycogen, blood lactic acid, and again liver glycogen. If, on the other hand, the liver is unable to form glycogen (or glucose) from lactic acid, this cycle would be interrupted as soon as the glucose molecule was deposited as muscle glycogen.

Mandel and Lusk (1) found that d-lactic acid, when administered to phlorhizinized dogs, leads to the excretion of extra glucose in the urine. This might be interpreted in the sense that, since lactic acid is convertible into glucose in the liver of the diabetic animal, it should also be able to form liver glycogen in the normal animal. However, the literature is contradictory on this point. Röhmann (2) states that ammonium and sodium lactate, when fed to rabbits fasted previously for 3 to 4 days, leads to glycogen deposition in the liver. Parnas and Baer (3) concluded that glycogen synthesis occurred during perfusion of the isolated turtle liver with sodium lactate. Barrenscheen (4), on the other hand, reported negative results in perfusion experiments on isolated livers of rabbits and dogs. Izume and Lewis (5) injected sodium lactate subcutaneously into two fasting rabbits and observed glycogen deposition in the liver. Takane (6) in Meyerhof's laboratory, obtained carbohydrate synthesis from sodium lactate which was added to sections of liver tissue suspended either in Ringer's solution

or blood serum. It appears from a remark on p. 417 of Takane's paper that glucose rather than glycogen was formed from the added sodium lactate, which this author ascribes to the special conditions of his *in vitro* experiments. Abramson, Eggleton, and Eggleton (7), working on dogs anesthetized with ether and amytal, were unable to demonstrate glycogen synthesis in the liver following intravenous injection of sodium *r*-lactate, though the injection of glucose, under the same conditions, led to glycogen formation in the liver.

The writers became interested in the question of glycogen formation from lactic acid when it was found that epinephrine when injected into 24 hour fasting rats, causes a simultaneous disappearance of muscle glycogen and an increase in liver glycogen in approximately equivalent amounts (8). It was suggested that part of the disappearing muscle glycogen was converted into liver glycogen with lactic acid as an intermediary stage, since there was no other obvious source for the newly formed liver glycogen and since carbohydrate oxidation was too low to account for the muscle glycogen which disappeared. In view of these results the experiments reported in the present paper were undertaken.

Meyerhof and Lohmann (9) found a marked difference in the utilization of *d*- and *l*-lactic acid in isolated mammalian tissues. Whereas *d*-lactic acid, when added to sections of liver, kidney, and brain tissue, increased the respiration, *l*-lactic acid had a doubtful effect. Liver tissue was able to synthesize carbohydrate from *d*-lactic acid but hardly from *l*-lactic acid.

The rate at which sodium *d*-lactate could be infused intravenously into rats without causing an appreciable rise in the lactic acid content of blood and urine was 95 ± 5 mg. per 100 gm. of body weight per hour (10). When the experiments were repeated with sodium *r*-lactate, a considerable amount of lactic acid was excreted in the urine (10). This suggested that there was also a difference in the utilization of the *d*- and *l*-lactic acid in the intact animal. It was therefore decided to use both forms of lactic acid in the investigation of glycogen formation in the liver and to include observations about the rate of absorption from the intestine and lactic acid content of blood and urine.

Methods.

The experiments were made on male rats after a fasting period of 24 hours. The weight of the animals at the time of feeding

varied between 100 and 160 gm.   Sodium *d-*, *r-*, or *l-*lactate was fed by stomach tube in the manner described in a previous paper (11). The amount of fluid introduced was 2.2 cc.   In some experiments 2.5 to 3.5 cc. of a 6 per cent solution of free lactic acid were fed without ill effects.   When sodium lactate was injected subcutaneously, three equal doses were given with an interval of 1 hour between each injection.   When it became desirable to feed a constant amount of lactic acid per unit of body weight, the following procedure was adopted.   The concentration of the lactic acid in the stock solution was determined by titration with phenolphthalein as indicator and by the Friedemann, Cotonio, and Shaffer method (12) with very satisfactory agreement.   The desired amount of lactic acid solution was delivered from a burette into a small beaker.   After addition of a small drop of methyl red, the solution was neutralized with 20 per cent NaOH.   The fluid in the beaker was drawn up with a syringe and injected through the catheter into the stomach.   Beaker, syringe, and catheter were then washed out with a small quantity of water. All animals were killed 3 hours after the administration of lactic acid.

Blood for duplicate lactic acid determinations was collected after decapitation.   The muscular movements during the collection of blood make the lactic acid values slightly too high, because, when blood is collected in the same manner from animals under amytal anesthesia, lower values for blood lactic acid are found.   The average for six determinations on normal rats under amytal anesthesia was 26.8 mg. per cent (10), while in the present series of determinations in Table I the average was 41.5 mg. per cent.   Urine was collected by placing the small wire screen cages, in which the animals were kept, on plates.   Urine remaining in the bladder after the death of the animals was added to that voided spontaneously.   The urine was made up to a volume of approximately 20 cc. in a 25 cc. volumetric flask, and 2.5 cc. each of 10 per cent $CuSO_4$ and 5 per cent $Ca(OH)_2$ were added in order to remove interfering substances.   After 30 minutes standing the solution was filtered and analyzed.   Extraction of the lactic acid from the urine with ether was found unnecessary, since the direct method gave only slightly higher values than after ether extraction.

After collection of blood, the liver was removed as quickly as

possible, frozen with CO_2 delivered from a tank, weighed in the frozen state, cut into small pieces, and introduced into boiling 60 per cent KOH.   Glycogen was determined according to Pflueger's method.   After hydrolysis of the glycogen with 2.2 per cent HCl, neutralization, and filtration, sugar was determined by means of the Bertrand method.   If less than 10 mg. of sugar was present in 20 cc. of the solution to be analyzed, a known amount of glucose was added before the determination was carried out.

For the determination of the amount of lactic acid absorbed from the intestine, a method was used which had been worked out previously for the determination of sugar absorption (11). The difference between the amount of lactic acid fed and the amount of lactic acid recovered from the whole intestinal tract represents the amount of lactic acid absorbed.   It is necessary to apply a slight correction because the intestine of a rat weighing 100 gm. contains on an average 10.9 mg. of lactic acid (Table I). After the death of the animal, the esophagus was tied and the whole intestinal tract was removed and placed in a beaker.   Stomach and intestines were cut open and extracted with repeated portions of hot water on a water bath at 100°.   The washings were poured into a 250 cc. volumetric flask, and before being made up to the mark, 12 cc. of colloidal iron and a few drops of a saturated solution of sodium sulfate were added.   An aliquot part of the filtrate was treated with copper sulfate and lime in order to remove interfering substances.   The final filtrate used for the lactic acid analysis was water-clear.   Control experiments showed that lactic acid is not included in the colloidal iron precipitate.

All lactic acid determinations were carried out by the Friedemann, Cotonio, and Shaffer method (12).   When this method was first used, and occasionally later, analyses of zinc lactate solutions containing known amounts of lactic acid were made.   The recovery of lactic acid was of the same magnitude as reported by the above authors.   Recently, the modification of Kendall and Friedemann (13), in which the permanganate used for the oxidation is substituted by colloidal manganese, was used with equal success.

Preparation of d- and l-Lactic Acid.[1]

Pederson, Peterson, and Fred (14) found that certain bacteria produce only the *d* form of lactic acid from glucose.   Dr. Peter-

[1] The older designation of the sarcolactic acid as *d*-lactic acid is used in this paper.

son kindly consented to isolate a sufficient quantity of *d*-lactic acid from the bacteria cultures for our purpose. His method of preparation was as follows: The fermented media, glucose-yeast water, were evaporated to a small volume, acidified, and the lactic acid extracted with ether. The acid was converted into the zinc salt by boiling with zinc carbonate. A sample for analysis gave 13.1 per cent water of crystallization. The zinc salt was dissolved in water, acidified, and extracted with ether. After the ether was removed, the solution was decolorized with norit and concentrated to about 38 per cent of lactic acid. A sample was converted into the zinc salt and an analysis gave 12.6 per cent water of crystallization. A sample of zinc lactate prepared by us 18 months later from the same *d*-lactic acid solution gave the following analytical results:
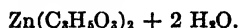
$Zn(C_3H_5O_3)_2 + 2 H_2O.$
    Found. H_2O, 13 06 per cent; ZnO, 29.09 per cent.
    Calculated.   " 12 89 "   "   " 29.11 "   "

(The racemic zinc lactate, which crystallizes with 3 molecules of water, yields 18.17 per cent H_2O and 26.73 per cent ZnO.) The rotation of a 2.5 per cent solution of the water-free salt in a 2.2 dm. tube was $\alpha = -0.45°$. $[\alpha]_D^{25} = -8.2°$. The magnitude of rotation of the *d*- and *l*-zinc lactate depends very much on the concentration. Jungfleisch and Godchot (15) reported for the same concentration (2.5 per cent of the water-free salt) $-8.0°$. Meyerhof and Lohmann (9) found for the *l*-zinc lactate $+8.1°$ and Neuberg (16) $+8.2°$.

The *l*-lactic acid was prepared according to Irvine's method (17), consisting of a resolution of the racemic acid with morphine. A concentrated solution of c. p. lactic acid was diluted to 20 per cent and was heated for 6 hours under a reflux condenser in order to destroy the anhydride. The hot solution was neutralized with morphine, filtered, and allowed to crystallize. A second crop of morphine *l*-lactate was obtained upon concentration of the mother liquor.[2] The salt was recrystallized from 50 per cent alcohol, dissolved in water, and decomposed with ammonia. After filtration and acidification, the free lactic acid was extracted with

[2] Up to this point, the preparation was carried out by Dr. Pucher of the Department of Biochemistry of the University of Buffalo.

ether in a continuous extraction apparatus. After the ether was removed, the solution was concentrated to 20 per cent of lactic acid. A sample was converted into the zinc salt by boiling with $ZnCO_3$ and was analyzed with the following results.

Air-dry (20 hours at 37°)...... 0.3146 gm. $Zn(C_3H_5O_3)_2 + 2H_2O$.
Heated for 4 hours at 120°.... 0.2737 "
Difference.................... 0.0409 " = 13.00 per cent H_2O.
Air-dry before ashing......... 0.2649 "
After ashing.................. 0.0778 " = 29.37 per cent ZnO.
$[\alpha]_D^{25}$........................ + 8.0° (c = 2.5 per cent of water-free salt, l = 2.2 dm., α = + 0.44°).

TABLE I.

Glycogen Content of Liver of 24 Hour Fasting Rats.

Average body weight 116 ± 10 gm.

Per 100 gm. body weight.			Liver glycogen.	Blood lactic acid.
Weight of liver.	Glyogen in liver.	Lactic acid in intestine.		
gm.	*mg.*	*mg.*	*per cent*	*mg. per cent*
3.73	4.2	12.2	0.11	47.0
3.21	2.1	8.3	0.06	46.5
3.35	2.8	12.0	0.08	34.8
3.40	1.8	10.7	0.05	43.4
3.46	4.0	11.2	0.11	42.5
3.43	2.9	11.0	0.08	35.1
3.25*	1.9		0.06	
3.18*	7.7		0.24	
3.38	3.4	10.9	0.10	41.5

* Fed 2.5 cc. of saline; killed 3 hours later.

Results.

The rats were subjected to a fasting period of 24 hours before lactic acid was fed in order to reduce the liver glycogen to a low level. The glycogen content of the liver of 24 hour fasting rats was determined on a series of eight control rats (Table I). The average was 0.1 per cent liver glycogen, or, since the liver weight was 3.38 per cent of the body weight, 3.4 ±1.4 mg. of glycogen for the liver of a 100 gm. rat. In previous determinations on 24 hour

fasting rats the average for sixteen experiments was 0.2 per cent or 7 ±2 mg. of liver glycogen per 100 gm. of rat (8). The values found by Macleod and collaborators (18) on rats fasted previously for 24 hours are in substantial agreement. In forty-eight experiments the average glycogen content was 0.16 per cent. The liver weight was not recorded, but assuming it to be on an average the same as in our experiments, this would correspond to 5.4 mg. of liver glycogen per 100 gm. of rat. It need hardly be pointed out that the remarkable constancy of the liver glycogen of 24 hour fasting rats makes such animals well suited for a study of glycogen formation. This rules out many uncertainties which are met with when glycogen formation in the liver of larger species is investigated. The possibility of determining absorption in the rat is a further advantage.

A comparison of the data in Tables I and II shows that the liver is able to form glycogen from lactic acid. There was only a small difference in the amount of glycogen formed during absorption of *d*- and *r*-lactate (on an average 53 mg. against 41 mg.). It would, however, be wrong to conclude from this that *l*-lactic acid is able to form liver glycogen as rapidly as *d*-lactic acid. This is not the case, as will be shown later. In order to explain the small difference between *d*- and *r*-lactate, the absorption from the intestine must be taken into consideration. The rats receiving *d*-lactate absorbed on an average 89.7 mg., while the rats receiving inactive lactate absorbed 115.1 mg., one-half of which (57.5 mg.) is *d*-lactic acid. The difference between the absorption of 89.7 and 57.5 mg. is not great enough to affect appreciably the rate of glycogen formation in the liver. The same is true for the experiments in which free *d*-lactic acid was fed, where the average absorption amounted to only 62.7 mg., while the amount of glycogen formed in the liver was 43 mg.

The percentage of absorbed lactic acid which is retained as glycogen in the liver is surprisingly high (Table II). It amounted on an average to 61.8 per cent when sodium *d*-lactate was fed and to 72.2 per cent when free *d*-lactic acid was given. In two cases, more than 95 per cent of the absorbed lactic acid was retained in the liver as glycogen. The percentage retention after feeding racemic lactate is only 34.2 per cent, because one-half of the lactic acid absorbed, namely the *l*-lactic acid, forms practically no

liver glycogen. Of glucose, fructose, and dihydroxyacetone 18, 38, and 21 per cent respectively of the amounts absorbed are retained as liver glycogen (19). On a percentage basis *d*-lactic acid is therefore a better glycogen former in the liver than any of these three sugars. It should be mentioned however that sodium lac-

TABLE II.

Glycogen Content of Liver 3 Hours after Lactic Acid Feeding.

Per 100 gm. body weight.				Liver glycogen.	Liver glycogen in per cent of amount absorbed.	Blood sugar.	Remarks.
Amount fed.	Amount absorbed.	Weight of liver.	Glycogen in liver.				
mg.	*mg.*	*gm.*	*mg.*	*per cent*		*mg. per cent*	
89	69 6	3.31	66 7	2.01	95 8	115	Sodium *d*-lactate.
116	86 7	3.20	36 8	1.15	42 4	100	Average body
125	90.1	3 28	57.8	1.76	64.1	113	weight, 145 ± 7
138	112.4	3 76	50.4	1.34	44.8	108	gm.
117	89 7	3 39	52.9	1.56	61.8	109	
148	84.0	3 05	25 0	0.82	28 8	105	Sodium *r*-lactate.
172	91.3	3 06	24.8	0 81	27.2	96	Average body
251	135.2	3.74	54 6	1 46	40.4	116	weight, 150 ± 9
267	150.0	3.65	58.8	1.61	39.2	108	gm.
209	115.1	3 37	40.8	1.18	34.2	106	
98		3.28	32 1	0.98			*d*-Lactic acid. Average body
102	43.1	3 65	34.3	0 94	79.5		weight, 147 ± 9
112	46.5	2.76	24.0	0.87	51.6		gm.
120	61.0	3.68	59 2	1.61	97.0		
150	84.2	4.10	50.8	1.24	60.3		
210*	79.0	3.55	57.2	1.61	72.3		
132	62.7	3.50	42.9	1.21	72.2		

* One-half neutralized with NaOH.

tate is absorbed approximately 7 times more slowly than glucose and 3½ times more slowly than fructose.

The blood sugar level is hardly changed during lactic acid absorption and the same is true following subcutaneous injection of lactate. This confirms the work of Janssen and Jost (20), Riegel (21), and Abramson, Eggleton, and Eggleton (7), who in-

jected sodium lactate intravenously. Izume and Lewis (5) state that sodium lactate in doses less than 2.0 gm. per kilo did not induce any appreciable hyperglycemia in fasting rabbits, while larger doses produced an increase in blood sugar.

The mechanism of absorption of lactic acid needs further investigation. In marked contrast to glucose and other sugars (11), the rate of absorption of sodium lactate and of free lactic acid depends on the amount fed. This is shown in Table II, in which the experiments are arranged according to the amount fed. Another striking difference exists between the absorption of sugars and lactic acid. Whereas isomeric sugars are absorbed at widely different rates from the intestine, for instance, mannose is absorbed 5 times more slowly than glucose (11), d- and l-lactate are absorbed at nearly the same rate (Table III). There is still one point which should be mentioned in connection with the experiments in Table II. For an equal amount fed, free lactic acid is absorbed more slowly than sodium lactate, but this is probably due to the acid reaction in the former case rather than to an intrinsic difference.

In order to afford a better comparison between optically active and inactive lactate, a standard amount of lactic acid was fed in all further experiments, namely 170 mg. per 100 gm. of body weight (Table III). This led to the absorption of nearly the same amounts of lactic acid in the three cases, the average being 111 mg. for d-lactate, 124 mg. for l-lactate, and 108 mg. for r-lactate. For an equal amount absorbed, r-lactic acid forms definitely less liver glycogen than d-lactic acid (26.1 mg. against 43.8 mg.). This is due to the fact that glycogen formation from l-lactic acid is almost entirely absent. It will be noted in Table III that the livers of the rats receiving l-lactate contained 10 ± 2.8 mg. of glycogen, while the livers of the control rats in Table I contained 3.4 ±1.4 mg. This is perhaps not an entirely negative result, though the difference is very slight indeed. The l-lactic acid used in these experiments, on the basis of the analyses made, is regarded as sufficiently pure to exclude an appreciable admixture of d-lactic acid, which, if it were present, would account for the small amount of liver glycogen formed. It is possible therefore that l-lactic acid is able to form liver glycogen at a very slow rate.

d- and *l-*Lactic Acid

Another striking difference between *d-* and *l-*lactic acid is found when the figures for blood and urine lactic acid are compared (Table III). Whereas during 3 hours of absorption of *d-*lactate only 0.5 mg. of lactic acid is excreted, 36.5 mg. appear in the urine

TABLE III.

Comparison of d-, l-, and r-Lactic Acid.

170 mg. of lactic acid per 100 gm. of body weight were fed in each case. The rats were killed 3 hours after the feeding.

Per 100 gm. body weight.				Liver glycogen.	Liver glycoogen in per cent of amount absorbed.	Blood lactic acid.	Remarks.
Weight of liver.	Amount absorbed.	Amount excreted.	Glycogen in liver.				
gm.	mg.	mg.	mg.	per cent		mg. per cent	
3 78	108	0 4	52 0	1 37	48 1	43 6	Sodium *d-*lactate.
3 59	103	0 4	56 5	1 57	54 8	53 8	Average body
3 60	118	0 6	32 6	0 91	27 6	63 7	weight, 110 ± 9
3 58	116	0 7	34 2	0 95	29 4	54 6	gm.
3 64	111	0 5	43 8	1 20	39 6	53 9	
3 48	104	27 9	8 6	0 24		113 8	Sodium *l-*lactate.
3 14	132	28 6	12 7	0 40		78 9	Average body
3 13	126	49 0	5 5	0 18		81 0	weight, 103 ± 3
3 21	135	41 4	13 2	0 41		87 1	gm.
3 77	113	41 9	12 5	0 33		65 5	
3 32	134	30 5	7 5	0 23		93 1	
3 34	124	36 5	10 0	0 30		86 6	
3 28	107	1 2	22 5	0 68	21 0	53 2	Sodium *r-*lactate.
3 26	96	1 6	35 1	1 08	36 5	68 2	Average body
3 10	105	0 9	30 8	1 00	29 3	75 0	weight, 107 ± 5
3 35	123	2 8	16 0	0 47	13 0	83 2	gm.
3 25	108	1 6	26 1	0 80	24 9	70 0	

when *l-*lactate is fed. This corresponds to an excretion of 29.4 per cent of the amount absorbed. The increase in blood lactic acid after the *l-*lactate feeding corresponds to a retention of 18 per cent of the amount absorbed, if it is assumed that the blood lactic acid is in equilibrium with 50 per cent of the body weight.

This leaves roughly 50 per cent of the amount absorbed or 62 mg. of *l*-lactic acid which, presumably, were utilized in the body in the course of 3 hours. Since *d*-lactate may be injected intravenously at a rate of 95 mg. per 100 gm. of rat per hour without causing an appreciable increase in blood lactic acid, or excretion in the urine (10), *l*-lactic acid is utilized approximately 4 times more slowly in the rat than *d*-lactic acid. A comparison of the experiments with *d*- and *r*-lactate in Table III, when based on excretion in the urine, does not reveal that there exists such a marked difference in the utilization of *d*- and *l*-lactic acid. This is due to the fact that after *r*-lactate feeding only half as much *l*-lactic acid is absorbed as after *l*-lactate feeding. In the latter case the rate of absorption of *l*-lactic acid was found to be 50 per cent higher than the rate of utilization. In the former case the rate of absorption does not exceed the rate of utilization and consequently the excretion in the urine is very small.

It remains to be determined how far a kidney factor might be involved in the different utilization of *d*- and *l*-lactic acid. Hewlett, Barnett, and Lewis (22) found in men that the threshold for lactic acid excretion is between 30 and 40 mg. per cent of blood lactic acid. This coincides fairly well with the values observed on rats under amytal anesthesia, when *d*-lactate was infused intravenously (10). As stated under "Methods," the lactic acid values in the present paper are approximately 15 mg. per cent too high, because muscular movements during the collection of blood were not abolished by an anesthetic, but this does not affect the following considerations. In Table III the average blood lactic acid after *d*-lactate feeding was 12.4 mg. higher than that of the control rats in Table I. Since after *r*-lactate feeding only half as much *d*-lactic acid is absorbed, it might be assumed that the increase in blood lactic acid was almost entirely due to *l*-lactic acid. This would give a concentration of 28.5 mg. per cent of *l*-lactic acid in the blood, at which level no appreciable quantity of lactic acid is excreted in the urine. After *l*-lactate feeding the average blood lactic acid was 45.1 mg. per cent higher than that of the control rats, and at this concentration in the blood lactic acid was excreted in the urine. The threshold for the excretion

of *l*-lactic acid is therefore not much different from that of *d*-lactic acid.

Experiments in which sodium *d*-lactate was injected subcutaneously are summarized in Table IV. A smaller amount of liver glycogen was formed than after *d*-lactate feeding. This may be

TABLE IV.

Glycogen Content of Liver after Subcutaneous Injection of Sodium d-Lactate.

160 mg. of lactic acid per 100 gm. of body weight, divided into three doses, were given.

Per 100 gm. body weight.			Liver glycogen	Blood lactic acid.	Blood sugar.
Weight of liver.	Amount excreted	Glycogen in liver			
gm.	*mg.*	*mg*	*per cent*	*mg. per cent*	*mg. per cent*
3 51	2 8	26 6	0 76	56 1	101
3 34	2 9	16 4	0 49	70 2	102
3 21	1 3	14 0	0 44	69 5	111
3 24	1 6	29 5	0 91	48 8	108
3 22	0 8	28 0	0 87	43 1	
3 25	2 9	28 4	0 88	46 2	
3 29	2 0	23 8	0.72	55 6	105

due in part to the fact that absorption from the subcutaneous tissue was not completed when the animals were killed.

DISCUSSION.

The main result of the present investigation is that *d*-lactic acid can be deposited as liver glycogen and that it is utilized several times faster in the rat than *l*-lactic acid. Also, the levo isomer is hardly able to form liver glycogen. This is another example of the discrimination of the body cells between two optical isomers. As stated in the introduction, Meyerhof and Lohmann (9) obtained the same results on isolated tissues of the rat. It is possible that narcosis abolishes the faculty of the liver to synthesize glycogen from lactic acid, because Abramson, Eggleton, and Eggleton (7) obtained negative results with dogs under ether and amytal anesthesia. Another contributory factor was probably the strong

alkalosis which they produced in their animals by intravenous administration of sodium lactate. The fact that glucose was still able to form liver glycogen under these abnormal conditions does, of course, not prove that lactic acid is unable to do so under more physiological conditions. The same authors state that there is no marked difference in the utilization of d- and l-lactic acid in the dog. However, they performed most of their experiments with r-lactic acid and did not compare d-and l-lactic acid directly. In the present experiments in which samples of d- and l-lactic acid of known purity were used, the difference in utilization was very marked.

The demonstration of glycogen synthesis in the liver from lactic acid links together some recently established experimental results. Himwich, Koskoff, and Nahum (23) found on decerebrate dogs, by an analysis of the arterial and venous lactic acid content of various organs, that the main site of lactic acid formation was the muscle, while the organ chiefly concerned with the removal of lactic acid from the blood was the liver. It seemed very probable that the liver formed glycogen from the lactic acid escaping from the muscles. Olmsted and Coulthard (24) actually observed a prolonged increase in liver glycogen in decerebrate cats. They explained this by a new formation of glycogen from an unknown carbohydrate existing in the body, but what actually took place was a conversion of muscle glycogen via lactic acid into liver glycogen. Epinephrine injections, which cause a disappearance of muscle glycogen in normal rats, also lead to glycogen formation in the liver from lactic acid (8). Geiger and Schmidt (25) showed recently that extra sugar in phlorhizinized dogs following epinephrine injections can be accounted for by the muscle glycogen which disappears. They failed, however, to realize that it was lactic acid and not glucose which was carried away by the blood stream to be converted into glucose in the liver.

Formation of liver glycogen from lactic acid is thus seen to establish an important connection between the metabolism of the muscle and that of the liver. Muscle glycogen becomes available as blood sugar through the intervention of the liver, and blood sugar in turn is converted into muscle glycogen. There exists

therefore a complete cycle of the glucose molecule in the body, which is illustrated in the following diagram.

```
                  ┌──────→ ── Blood ──────→──┐
                  │             glucose        │
                  │                            │
            Liver │                            │ Muscle
          glycogen│                            │ glycogen
                  │                            │
                  │                            │
                  └── ──←──── Blood ──────←──┘
                             lactic acid
```
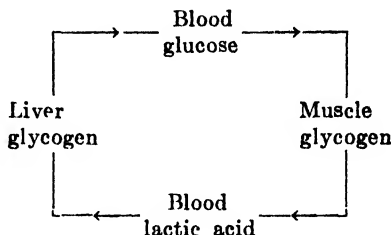
Epinephrine was found to accelerate this cycle in the direction of muscle glycogen to liver glycogen and to inhibit it in the direction of blood glucose to muscle glycogen; the result is an accumulation of sugar in the blood. Insulin, on the other hand, was found to accelerate the cycle in the direction of blood glucose to muscle glycogen, which leads to hypoglycemia and secondarily to a depletion of the glycogen stores of the liver. It will be investigated to what extent this cycle plays a rôle in the preservation of liver glycogen and hence of a normal blood sugar level during fasting. There is also a possibility that other hormones besides epinephrine and insulin influence this cycle.

SUMMARY.

1. Sodium *d*-lactate, when fed by mouth or injected subcutaneously, leads to glycogen deposition in the liver. Sodium *l*-lactate, though it is absorbed at the same rate from the intestine as the dextro isomer, hardly forms any liver glycogen. Of *d*-lactate 40 to 95 per cent of the amount absorbed in 3 hours is retained as liver glycogen.

2. Of *l*-lactate 30 per cent of the amount absorbed is excreted in the urine, while no excretion occurs during *d*-lactate absorption. It is estimated that *l*-lactic acid is utilized 4 times more slowly in the rat than *d*-lactic acid.

3. The rôle of the cycle, liver glycogen → blood glucose → muscle glycogen → blood lactic acid → liver glycogen, as an important phase of carbohydrate metabolism, is emphasized.

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THE AMIDE NITROGEN OF BLOOD.

IV. A METHOD OF EXPRESSING RESULTS BASED UPON THE PROTEIN CONTENT OF BLOOD.

By SIDNEY BLISS.

(From the Department of Biochemistry, McGill University, Montreal.)

In a former communication (1) it was pointed out that blood concentration changes had been followed by the determination of the total nitrogen of blood.

Further studies of the effect of changes in blood concentration indicate that the method of expressing results used in the former communication must be changed from the conventional basis of the number of mg. of the substance per 100 cc. of blood to that of the number of mg. of amide nitrogen per 100 gm. of blood protein.

This is done in the following way. Analysis of a sample of blood yielded the following data.

Found.   Total protein N.   3 20 per cent.
             Amide N.   150 mg. amide N per 100 cc. blood.

Calculation.—In such a case 100 cc. of blood contain 6.25 × 3.20, or 20 gm. of protein. To express the amide nitrogen content on the basis of 100 gm. of blood protein in this instance, the 150 mg. of amide nitrogen found in 100 cc. of blood were the amount of amide nitrogen that was carried by 20 gm. of protein, and hence it is multiplied by 5 in order to express the amide nitrogen in terms of the amount of it carried by 100 gm. of protein. In this case the value is 5 × 150, or 750 mg. of amide nitrogen per 100 gm. of blood protein.

To determine the total protein nitrogen of blood one may use 1 cc. of whole blood for a macro-Kjeldahl determination, making the necessary correction for the non-protein nitrogen of blood, which, however, amounts to but 1 per cent of the total nitrogen. We have found that for a large number of determinations it is

convenient to use an adaptation of Folin's micro digestion method (2), followed by Nesslerization.   We have used the latter method in the following way.

In the method already described for the estimation of the amide nitrogen content of blood (3), there is available a solution of the proteins of blood which serves as a starting point for the estimation of the total protein nitrogen of blood.   In the estimation of the amide nitrogen content of blood, one uses for acid hydrolysis 2 cc. of a 1:10 dilution of blood proteins obtained by dissolving the tungstic acid precipitate in 0.9 N NaOH.   This dissolved protein is merely diluted further, and an appropriate aliquot taken for total nitrogen estimation as follows:

Into a 20 cc. volumetric flask, nearly filled with distilled water, discharge 2 cc. of the dissolved protein precipitate of which 2 cc. were also used for acid hydrolysis in the method for the estimation of the amide nitrogen content of blood.   Make up to mark and mix.   1 cc. of this 1:100 dilution of blood protein is now digested in a 200 × 25 mm. Pyrex tube with 1 cc. of the 1:1 dilution of Folin's sulfuric-phosphoric acid digestion mixture (4).   When the digestion is finished, allow 90 seconds for cooling and then add 2J cc. of distilled water.   Cool the contents of the tube and transfer to a 50 cc. volumetric flask and rinse the tube with successive small quantities of water until the final volume of fluid in the 50 cc. volumetric flask amounts to about 30 cc.   A similar volumetric flask is used for the preparation of the standard, with 0.3 mg. of nitrogen (as ammonium sulfate) and the same amount of digestion mixture as was used to digest the protein, the whole being made up, as in the case of the unknown flask, to about 30 cc.   Both flasks are now Nesslerized with 15 cc. each of Nessler solution, made up to the mark, and mixed.   If the unknown matches the standard, the blood contains 3 per cent protein nitrogen.   To calculate any value, divide the reading of the standard by the reading of the unknown and multiply by 3 to obtain the number of gm. of protein nitrogen per 100 cc. of blood.

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THE COMPOSITION OF THE BODY FLUIDS OF ELASMOBRANCHS.*

By HOMER W. SMITH.

WITH THE ASSISTANCE OF JAMES P. BAKER AND HERBERT SILVETTE.

(*From the Department of Physiology, University of Virginia, University, and the Mt. Desert Island Biological Laboratory, Salisbury Cove, Maine.*)

In the course of experiments ranging over several years we have had occasion to examine the blood and other body fluids of a number of animals, our interest centering largely on the question of the possible rôle of secretion, as contrasted with simple filtration from the plasma, in the formation of these fluids. This investigation has not included any extensive series of experiments, and we wish only to report the inorganic and nitrogenous constituents of the body fluids as removed from living animals under, as nearly as possible, natural conditions.

The presence of extraordinary amounts of urea in the blood and tissues of elasmobranchs was demonstrated by Staedeler and Frerichs (1). Bottazzi (2) observed that the osmotic pressure of elasmobranch blood is about the same as that of sea water, and he was puzzled by the fact that there is so much less salt in the blood than in sea water. It remained for Rodier (3) to point out that the urea makes up the difference in osmotic pressure, which may be a third to one-half the whole.

Numerous investigators have since determined the urea content of the blood in a large number of species of the elasmobranchs, and from their observations it may be concluded that a high blood urea is a phyletic character of the orders Selachii and Batoidei. This high urea concentration presents an interesting problem from the point of nitrogen metabolism and urinary excretion, but it is

not with this constituent of the blood that this paper is primarily concerned.

Methods.

The carbon dioxide content was determined by the method of Van Slyke and Neill (4); chloride by the method of Van Slyke (5), ether being added prior to titration to insure a sharp and permanent end-point; and hydrogen ion concentration with quinhydrone and a gold electrode designed for use with small quantities of fluid and to prevent loss of CO_2 (Bülman (6)). The inorganic and nitrogenous constituents were determined on a trichloroacetic acid filtrate obtained by adding 1 volume of serum, *etc*, to 1 volume of 20 per cent trichloroacetic acid solution plus 3 volumes of water. Trichloroacetic acid was used as the protein precipitant because it is possible to determine the inorganic constituents as well as most of the nitrogenous constituents on one filtrate. Sulfate was determined in the filtrate either by the method of Denis (7) or by benzidine precipitation (Fiske (8)) after adjusting the reaction to approximate neutrality; phosphate was determined by the Briggs modification of the Bell-Doisy method (9); sodium, potassium, calcium, and magnesium were determined on the filtrate after ashing and redissolving in water; potassium by the method of Kramer and Tisdall (10), calcium by the method of Clark and Collip (11), and magnesium on the calcium-free filtrate by precipitation as magnesium-ammonium-phosphate and determination of the phosphate in an aliquot by the Bell-Doisy method (9). Sodium was determined by the method of Kramer and Gittleman (12). It is a routine practice to add 0.5 cc. of 10 per cent KOH to the sample prior to the addition of the potassium antimonate; this precipitates the magnesium and the sodium is subsequently thrown down upon this magnesium precipitate. We have never found that this substance, even when present in high concentrations, interferes with the determination of the sodium. The non-protein nitrogen and urea determinations were made on aliquots obtained by careful dilution; non-protein nitrogen either by the Folin-Wu method (13) or the Koch-McMeekin method (14). We believe that digestion of animal bloods can be carried out more completely by the latter method, possibly because the

process of digestion can be continued for 15 to 20 minutes. Ammonia was determined by extraction with permutit after neutralization of the filtrate with N NaOH and litmus paper, reextraction of the ammonia with alkali, and direct Nesslerization as described by Folin and Bell (15) for urine. When the ammonia concentration was low, as in serum, this method was supplemented by direct Nesslerization of the filtrate. No attempt was made to determine accurately quantities less than 1.0 mg. of NH_3 N per 100 cc. Urea was determined on the filtrate after careful neutralization with litmus paper and extraction of ammonia with permutit, if any ammonia was present. The urea was decomposed with urease in the presence of phosphate buffer, and the resulting ammonia determined by direct Nesslerization. In the determination of creatinine the filtrate was first neutralized with litmus paper and N NaOH, and then freshly prepared alkaline picrate solution added and the mixture compared with a suitable creatinine standard. Creatine was converted to creatinine by the Folin-Wu water bath procedure (13). An equal volume of N HCl was added to the filtrate and the mixture heated in the water bath for 2 to 3 hours. This was then neutralized with N NaOH before the addition of the alkaline picrate solution. All creatinine and creatine determinations on a group of fluids from one animal were made simultaneously, and a blank, made up to the same volume as the unknown, was included and the blank reading subtracted from the unknown. This is important where the quantities of creatinine or creatine are small.

The pericardial cavity of a medium sized barn-door skate (20 inches broad) contains from 6 to 10 cc. of fluid. This is colorless and has only a few cells in suspension. It is practically protein-free. The peritoneal cavity of a skate of this size may contain from 3 to 30 cc. of fluid, which is described here as perivisceral fluid. This is likewise colorless and as a rule has more cells in suspension than the pericardial fluid, but never contains more than a trace of protein. That these fluids are normal, and not pathological contents of these body cavities, is indicated by the fact that they are invariably present in greater or lesser quantities in freshly killed, normal animals. These fluids, as well as the

cerebrospinal fluid,[1] can be quickly removed from the live animal by aspiration into a Luer syringe after simple operative procedures exposing the respective cavities. Blood can be obtained most easily by aspiration from the venous sinuses dorsal to the heart.

The gland described as Marshall's gland, is variously designated in comparative anatomies as a "urinary bladder" and as a "sperm sac." Marshall (16) has shown that it contains neither urine nor sperm, but that it secretes a strongly alkaline solution apparently intended to protect the sperm from the acid urine in passing through the uterus of the female. (The bladder in the female empties into the top of the uterus. Fertilization is internal in these animals.)

<center>DISCUSSION.</center>

Inorganic analysis of the sera of the dogfish, *Acanthus vulgaris* and the sand-shark, *Carcharias littoralis*, have been made by Macallum (17). Our figures agree very well with those of Macallum, except that we find a greater range of variation in the potassium and calcium content, contrary to his expressed belief that this variation should be a very limited one. Macallum states that the "amount of ammonia present in the serum (of the dogfish) was found to be 0.1727 per cent, a concentration slightly greater than $N/10$ . . . . ." We have never found any significant quantity of ammonia in the blood of the dogfish, *Mustelus canis*, or other elasmobranchs, and we suggest that in the sample Macallum analyzed, ammonia had been formed by decomposition.

Concerning the urea in elasmobranchs Macallum remarks, "Their (urea and ammonia) occurrence in such concentrations in the blood plasma of sharks indicates how relatively inert in their elimination are the kidneys of these forms." From which it is inferred that he considers this urea to be a waste product of nitrogen metabolism as it is, apparently, in the mammals, and therefore something to be gotten rid of if the kidneys were capable of this task. We believe that there is ample reason to refute this view,

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[1] It has been suggested to us by Dr. E. K. Marshall, Jr., that the fluid removed from the skull cavity of fish may not be cerebrospinal fluid, for he has observed that in *Lophius* it appears to lie outside the meninges, and in both *Lophius* and the elasmobranchs it forms a clot after withdrawal from the body.

and look upon the urea as a metabolite specifically retained by these fishes for its osmotic pressure, a circumstance related to the reduced salt content of their blood and to the regulation, or absence of regulation, of the water distribution across the gill membranes. The persistence of the urea during fasting is one reason for this belief. This problem will not be considered in the present paper, however.

The results of our analyses are given in Table I, where the figures in bold face type indicate unusual concentrations of any constituent. The most noteworthy features shown by these analyses are:

1. Pericardial Fluid.—(*a*) This fluid is consistently more acid than the plasma in all species which we have examined. The acidity, moreover, is greater than we had previously supposed possible for a normal body fluid. Our pH values on the pericardial fluid range from 5.3 to 6.9, as compared with the serum value in the same animals of from 7.2 to 7.5. With the increased acidity there is a concomitant decrease in the concentration of bicarbonate. Though the fact cannot be shown by our data, we believe that the original CO_2 tension in all the body fluids is approximately the same as that of the blood, in which case, of course, the pH and bicarbonate content would vary inversely. (*b*) The calcium content of the pericardial fluid is consistently lower than that of the serum, ranging from one-seventh to one-half of the latter. (*c*) The potassium, on the other hand, is considerably higher in the pericardial fluid than in the serum, ranging from $1\frac{1}{2}$ to 5 times the latter. Our data suggest a possible inverse relationship between the potassium content and the calcium content. The remarkable nature of the pericardial fluid is shown in Table I, Fish 1, in which the pH is 6.86 as compared with 7.35 in the serum, the potassium content 4 times as great, and the calcium only one-seventh as great as in the serum.

2. Perivisceral Fluid.—(*a*) The perivisceral fluid is, in general, more acid than the pericardial fluid, but does not exceed the extreme value pH 5.3. The titratable acidity of this, and of the pericardial fluid (to pH 7.0) is very small, not more than 2 to 3 mM per liter. (*b*) The calcium content is generally less than that of the serum, though it may sometimes exceed the latter. (*c*) The potassium content is usually about the same as that of the

TABLE I.

Composition of Body Fluids of Elasmobranchs.

Fish No.		Na	K	Ca	Mg	Cl	SO_4	PO_4	CO_2	ΣB^+	ΣA^-	pH	Non-protein N	Urea N	NH_3 N	NH_2-N	Creatinine N	Creatine N
		mM per liter											Mg per 100 cc					
1	*Raja stabuloforis*, ♂.																	
	Plasma.	262	5.0	3.8	1.7	232	Trace.	0.7	6.0	278	239	7.35		1245				
	Cerebrospinal fluid.	262	3.8	3.0	0.9	244	"	1.0	5.5	274	251	(7.64)		1240				
	Perivisceral "	299	5.2	1.7	**21.7**	332	**13.9**	0.7	0.4	351	361	**6.15**		1309				
	Pericardial "	353	**21.0**	**0.5**	1.0	366	—	0.7	0.3	377	367	**6.86**		**900**				
	Marshall's gland.	484	5.2	Trace.	**0.0**	345	20.8	0.0	**91.0**	489	478	7.72		514				
	Gastric contents.	303	2.4			274	**105.0**	0.0		305	484			1245				
2	*Raja stabuloforis*, ♀.																	
	Plasma.	236	4.5	2.9	2.4	230	Trace.	0.7	7.16	251	238	7.63		1300				Trace.
	Perivisceral fluid.	274	6.4	3.8	**14.0**	324	"	0.0	0.3	315	324	**5.66**		1250				"
	Pericardial "		**22.0**	**0.8**	5.0							**6.40**						
	Bile.	322	5.7	4.2	3.9	212	Trace.	0.0	**19.1**	344	231	7.96		1250				Trace.
3	*Raja stabuloforis*, ♂.																	
	Plasma.	255	5.2	3.8	3.5	235	0.5	1.6	5.56	271	244	7.21		1308			0	
	Perivisceral fluid.	257		1.9	**16.1**	237	2.5	0.0				**6.05**		1346			0	
	Pericardial "	312	**17.8**	**0.5**	1.5			0.0				**6.07**		**1039**			0	
	Marshall's gland.					307	**31.0**	0.0	**115.0**					312			Trace	

The figures in bold face type signify an unusual concentration of any constituent. The figures in parentheses denote uncertain values.

4	*Raja stabuloforis, ♂.*													
	Plasma.	267		4.5	3.5	266	Trace.	0.9	5.6	7.33	1220			1.4
	Cerebrospinal fluid.	267	4.5	6 9		282	"		5.6	7.39	1200			0.8
	Periviseral "	304	20.2	0.9	20.0	344	5.3		0.5	5.40	1140			1.4
	Pericardial "	340	5 7	0.0	2.5	373	Trace.	0.0		6.35	1100			
	Marshall's gland.	475				292	35.8		101.0		470			
5	Sea water on Fish 1 to 4.	416	9.1	9 4	50 0	483	30.4		2.2	544 546	8 2			
6	*Raja diaphenes, ♀.*													
	Plasma.	237	6 8	5 1	3 5	227	3.1	1.4	5.6	261 240	7.26	1070 1052	0	9.4	0.5	1.4
	Cerebrospinal fluid.		6.8	2.3	20.0	188	0.4	0 9	4 6	7.15	681 555	0	2.6	0.7	0.8
	Periviseral "	145					14.0	0 9		196 217	6.35	758 692	8.6			1.4
	Pericardial "		6.2	10 9	5.3				3.7	208	6.19					
	Bile.	170									6.47					
7	*Raja diaphenes, ♀.*													
	Periviseral fluid.	166	5.6	4.0	8.0	188	2.0	0 5		195 193	5.3	837 737	10.0	0	0	0.7
8	N. Y. Harbor water on Fish 6 to 7.	206	4.6	4 5	22.6	225	13.2		2.0	251 253	8.0	.			
9	*Carcharias littoralis, ♂.*													
	Plasma.	258	5 5	5 0	2.3	228	0.3	2.5	9 3	278 246	7.20	1125 1080	Trace.	7.5	0.4	0.9
	Periviseral fluid.	276	8 9	3 6	25.0	306	1.4	0.6	Trace.	342 310	5.57	1015 996	12.9			1 2
	Pericardial "	290	10.1	2 2	2 3				"		5.30					
10	*Carcharias littoralis, ♂.*													
	Plasma.	275	5 4	6.0	2 3	241	0.7	3.4	11.2	297 259	7.52	1074 1045	0	5.6		0.6
	Cerebrospinal fluid.								10.5		7.60	890				
	Periviseral "		8.5	6.6	17.8		1.15				5.92		60.4			
	Pericardial "			3.5	3.1						5.57		3.3			

TABLE I—*Concluded.*

Fish No.		Na	K	Ca	Mg	Cl	SO₄	PO₄	CO₃	ΣB+	ΣA-	pH	Non-protein N	Urea N	NH₃ N	NH₂-N	Creatinine N	Creatine N
						mм per liter									Mg. per 100 cc.			
11	*Mustelus canis*, ♂.																	
	Plasma.	270	4 4	5 1	2 9	237	2 6	3 5	11 5	290	248	7 56	1180	1120	0	9.2		0 7
	Cerebrospinal fluid.								10 0			7 18						
	Pericardial "											6.98						
12	*Mustelus canis*, ♀.																	
	Plasma.	270	5 6	5 8	3 0	230	1 79	3 4	11 6	293	250	7 57	1070	1010	0	5 3		0.7
	Cerebrospinal fluid.	270	6 0	4 0	1 0	260	1 0	1 7	(7 7)	286	271	7 77	900	840	10.0	0		
	Perivisceral "											6.84			0 3			
	Pericardial "											6.91						
13	Sea water on Fish 9 to 12.	445	10 6	14 5	50 0	517	30 6	0 2	2 4	584	578	8 4	0					

serum. (*d*) Unlike the pericardial fluid, the perivisceral fluid is characterized by the frequent presence of large amounts of sulfate, magnesium, and ammonia.

The pericardial and perivisceral fluids may contain as much urea as the serum, but in some instances they contain considerably less. This unequal distribution of urea between the serum and these body fluids is all the more significant in view of the approximately equal distribution of this substance per kilo of water between the serum and tissues, as shown in Table II. This fact suggests that along with the hydrogen and bicarbonate ion, and with the potassium, calcium, magnesium, and sulfate, the urea concentration of these fluids may be the result of a "conditioning" secretion rather than of simple diffusion.

TABLE II.

Distribution of Urea between Body Fluids and Tissues.

Fish No.		Plasma.	Blood cells.	Skeletal muscles.	Liver.	Gonads.	Spleen.	Intestine.	Skin.	Gastric juice.	Cerebrospinal fluid.	Perivisceral fluid.	Pericardial fluid.
14	*Raja stabuloforis*, ♀.												
	H$_2$O per kilo, *gm*....	937	695	750	357	836	779	818	707	960	950	954	960
	Urea N per 100 gm.												
	H$_2$O, *mg*.........	880	960	900	980	868	829	855	1100	179	700	680	600

From the point of view of development the pericardial space is part of the cœlom, and it is commonly stated in text-books of comparative anatomy that in the elasmobranchs the two communicate by means of a pair of pericardio-peritoneal canals which penetrate the visceral aspect of the pericardium. The student is usually instructed to demonstrate these canals by pushing a fine wire or a stiff bristle through them. That the fluids in these two cavities do not freely intermingle is adequately demonstrated by the marked differences in composition of the pericardial and perivisceral fluids, but it is possible that the pericardial fluid is mechanically discharged into the cœlom · under special circumstances.

It should be noted that the pericardial space is generated,

figuratively, by the pushing of the heart into one side of a potentially symmetrical cavity bounded by the pericardium; hence this membrane lies between the pericardial fluid and the blood both in the heart and arteries, and in the tissues exterior to the heart. So this fluid of unusual composition must be considered a specific elaboration of the pericardium; and inasmuch as it appears impossible to explain its composition in terms of diffusion of plasma across any membrane familiar to us at present, we feel justified in applying to it the term secretion.

The composition of the perivisceral fluid is no less striking than that of the pericardial fluid. The parietal layer of the peritoneum covers the body cavity and the visceral layer is reflected over the viscera. In view of the enormous differences in composition between the perivisceral fluid and the serum, the conclusion is unavoidable that the peritoneum, like the pericardium, is endowed in the elasmobranchs with secretory capacities.

It is possible that the ammonia in the perivisceral fluid is the result of enzymatic decomposition of ammonia precursors occurring after the fluid is removed from the body, but since in several instances special precautions were taken to add trichloroacetic acid immediately, it appears that ammonia is present as such in the fresh fluid.

It may be remarked that the pericardial and perivisceral fluids in mammals are commonly looked upon as transudates serving merely to lubricate these cavities. In mammals, the quantities of these fluids normally present are small, in marked contrast to the case in the elasmobranchs and other vertebrates. It is noteworthy to find, in these fluids, such highly differentiated secretions, especially in view of their origin from apparently indifferent serous membranes. Taking all the constituents into consideration, the presumption is that a serous membrane may be endowed with secretory capacities approximating those of an alveolar gland. The structure of the latter may be related more to quantity production than to quality determination.

The presence of magnesium, sulfate, and ammonia in the perivisceral fluid strongly suggests an excretory function for the peritoneal cavity in these fishes. This possibility is supported by the presence of abdominal pores which open to the exterior near the anus. The presence of abdominal pores in fishes has been

investigated by a number of workers, though comparatively little has been done in the direction of demonstrating an excretory function for them. Their presence is usually detected by placing mercury in the abdominal cavity.

There is some question as to whether abdominal pores are to be considered as a primitive condition, or one secondarily acquired by the elasmobranch and other fishes. They are present in nearly all the Elasmobranchii, in the Crossopterygii, and in *Protopterus* and *Neoceratodus*, but absent in *Lepidosiren*. They are present in the Chrondrostei and Holostéi and a few species of Teleostei (Bles (18)).

Bles has emphasized that there is, apparently, a close correlation between the existence of open nephrostomes and the presence of abdominal pores in the elasmobranchs. Both open nephrostomes and abdominal pores are present in eight species which he listed; four species possessed open nephrostomes but no abdominal pores, while twenty-four species had abdominal pores, but the nephrostomes were lost early in development. He concluded from this correlation that " . . . . as they [abdominal pores] alternate on the whole in their distribution with the nephrostomes, they undertake in all probability the duties of the absent nephrostomes . . . . " and that " . . . . the body cavity of Fishes . . . . is to a great extent an excretory organ, as it certainly is in the early stages of the development of almost all vertebrates."

We have not been able to establish any connection between the presence or absence of perivisceral fluid and patency of the abdominal pores. In some instances it was thought that the pores were originally closed and were ruptured by the mercury, for it could be seen that the papillæ were gradually distended by the mercury, the distention growing great enough in the course of half a minute or so as apparently to cause rupture. In other instances one or both pores were apparently open, for the mercury escaped as soon as it was introduced into the abdominal cavity. Occasionally we have definitely observed the discharge of fluid from the pores on taking the animal out of sea water, especially if it was struggling. It seems probable, therefore, that the perivisceral fluid is discharged from the body at infrequent intervals through the abdominal pores. As was remarked above, the presence in it of

sulfate, magnesium, and ammonia in such large amounts definitely suggests an excretory function.

We have not had an opportunity to examine any of the elasmobranchs which do not at any time possess abdominal pores. (It may be noted that the Carchariidæ (*Carcharias littoralis* and *Mustelus canis*) and the Batoidei (*Raja diaphenes, eglenteria,* and *stabuloforis*) do not possess open nephrostomes in the adult.)

Except in so far as the pericardial fluid may be discharged into the abdominal cavity through the pericardio-peritoneal canals, there is no reason to believe that this fluid is an excretion. The absence of magnesium, sulfate, and ammonia also mitigates against this view.

3. Cerebrospinal Fluid.—This fluid possesses all the qualifications, on rather superficial analysis, to entitle it to the name "plasmadialysate." That is, the distribution of bicarbonate and of sodium, potassium, calcium, and magnesium between the plasma and the cerebrospinal fluid is such that the latter could conceivably come into existence by the diffusion of plasma through a membrane impermeable to the plasma proteins, and in this sense the cerebrospinal fluid of these fishes resembles the cerebrospinal fluid of mammals. The intervention of a membrane endowed with the specific capacity to condition the distribution of certain ions or substances need not, apparently, be involved in its formation. But our admittedly meager data can only suggest a dialysate nature for this fluid. Proof in this direction would require a much more exhaustive examination under a variety of conditions. On the other hand, such an examination might reveal departures from the composition of the plasma entirely contrary to this expectation. Possibilities in this direction are suggested by the difference in urea concentration in the plasma and the cerebrospinal fluid of Fishes 6, 10, and 14.

In conclusion it is noteworthy with reference to all these fluids that there is a reciprocal relation between the concentration of inorganic substances and urea; where the former are increased, the latter is diminished in amount. Osmotic pressures or freezing points were not determined, but the correspondence of the sum of urea molality and the estimated osmotic activity of the salts indicates that all the body fluids are approximately isotonic with the blood.

SUMMARY.

1. The pericardial and perivisceral cavities of the elasmobranch fishes contain fluids which are extremely acid and which may differ markedly from the plasma and from each other, in respect to urea, sodium, potassium, calcium, magnesium, sulfate, chloride, and bicarbonate.

2. An excretory function is possibly indicated in the case of the peritoneum and the perivisceral fluid, since the latter may be discharged through the abdominal pores.

3. The cerebrospinal fluid approximates a protein-free ultrafiltrate from the plasma.

4. Urea, which is a special metabolite in these fishes and not primarily a mode of nitrogen excretion, may occur in very different concentrations in the body fluids, although it is approximately equally distributed, per kilo of water, between the plasma and the tissues.

5. Emphasis is placed upon the secretory capacities of the pericardial and peritoneal membranes.

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THE EFFECT OF INSULIN ON THE DISTRIBUTION OF NON-PROTEIN NITROGEN IN THE BLOOD.*

BY STANLEY E. KERR AND VARTAN H. KRIKORIAN.

(From the Department of Biological Chemistry, American University of Beirut, Beirut, Syria.)

In view of Sokhey and Allan's report (1924) that insulin causes an increased excretion of nitrogen in the urine, it seemed significant to study the effect of insulin on the non-protein nitrogen compounds of blood. Recently Luck, Morrison, and Wilbur (1928) showed that insulin causes a decrease in the amino acid nitrogen of blood, and Kicch and Luck (1928) demonstrated satisfactorily that the decrease in amino acid nitrogen of the whole body is entirely accounted for by a corresponding increase in urea. In view of the fact that the present results are largely corroborative in nature, they are presented in the form of averages instead of being given in detail.

The experiments were performed on dogs fasted for 24 to 48 hours. Serum and corpuscles were analyzed separately, by the methods of Folin and Wu. In one series of experiments a comparison was made between normal blood and that taken during the period of extreme depression $2\frac{1}{2}$ to 5 hours after the insulin injections. The results, presented in Table I, show a decrease in amino acid nitrogen, with a correspondingly larger increase in urea nitrogen. In some of the experiments the rise in urea nitrogen was less than the fall in amino nitrogen, possibly due to a loss of urea in the urine. In other experiments, however, the increase in blood urea was as much as 10 times as great as the decrease in amino acid nitrogen, indicating that the increase in urea was not at the expense of the amino acids of the blood only, but of the tissues as well. The decrease noted in the undetermined fraction

of the non-protein nitrogen after insulin is of doubtful significance. The experimental procedure, including the initial bleeding, caused no change of significance, as shown by the control experiments. In another series of six experiments (not included in the tables), the creatinine of serum and corpuscles was shown to be entirely unaffected by insulin. Corpuscle creatine showed irregular changes, increasing slightly from an average normal of 1.06 mg. of

TABLE I.

Effect of Insulin on Distribution of Non-Protein Nitrogen in Blood.
Results are expressed in mg. per 100 gm.

Blood sample analyzed.	Glucose.		Non-protein N.		Urea N.		Amino N.		Undetermined N.		Remarks.
	Serum.	Corpuscles.	Serum.	Corpuscles.	Serum.	Corpuscles.	Serum.	Corpuscles.	Serum.	Corpuscles.	
Before insulin.	108.8	35.8	18.4	30.7	8.2	7.0	5.4	8.5	4.8	15.2	5 units insulin per kilo. Average of five experiments.
After "	42.2	24.0	18.2	30.7	11.2	8.8	3.9	7.4	3.1	14.5	
Before insulin.	96.2	35.7	20.8	32.9	12.0	9.6	5.2	8.1	3.7	15.2	10 units insulin per kilo. Average of five experiments.
After "	40.8	29.0	21.5	34.2	14.2	11.4	4.1	7.7	3.2	15.1	
Normal....... .	99.6	36.7	19.5	33.5	11.3	10.2	5.1	9.8	3.1	13.5	Controls. No insulin. Average of three experiments.
3 hrs. after 1st bleeding... ..	99.4	34.0	18.3	34.2	11.7	10.1	4.7	9.4	1.9	14.7	

nitrogen per 100 gm. to 1.24 mg. after insulin. These results are interesting in view of the report by Hill (1928) that creatine administration causes a fall in blood sugar concentration.

In one experiment (No. 14, Table II), whole blood was analyzed at frequent intervals over a period of 3 hours after the insulin, in order to detect changes which might take place before the occurrence of hypoglycemia. The results showed a slight increase in

amino acid nitrogen and a corresponding fall in urea nitrogen half an hour after the insulin, these changes being later reversed.

TABLE II.

Effect of Insulin on Urea and Amino Acid Nitrogen in Blood.

Results are expressed in mg. per 100 cc. of whole blood.

Experiment No.	Insulin dosage.	Interval after insulin.	Remarks.	Glucose.	Urea N.	Amino N.
	units per kg.	*min.*	•			
14	5	0	Male dog, 14 kilos, fasted 24	96.6	12.2	8.2
		35	hrs.	73.8	11.9	8.4
		60		71.0	12.3	7.0
		120	.	67.1	12.8	6.3
		180	Depressed.	58.7	11.7	5.9
		hrs.				
15	10	0	Male dog, 18.5 kilos, fasted 48	80.3	16.2	8.0
		1	hrs.	73.0	14.6	6.9
		2	Depressed.	72.2	14.6	6.7
		3	"	67.3	14.3	5.9
		4	"	54.0	14.9	5.9
		5	"	45.2	12.6	6.1
		6	"	48.1	12.8	6.3
		7	"	46.7	12.6	5.8
16	10	0	Male dog, 17 kilos, fasted 48 hrs.	87.0	13.7	7.7
		3	Greatly depressed. Barely able	59.5	14.6	6.1
		5	to walk.	52.1	12.5	5.6
		7		48.6	13.3	5.1
		9	Convulsions.	40.8	11.6	6.4
17	20	0	Male dog 10 kilos, fasted 24 hrs.	70.4	11.2	7.8
		3	Slightly depressed.	52.9	10.8	5.5
		5	Unable to stand.	39.2	10.6	5.4
		6½	Salivation, labored breathing.	37.2	9.0	6.0
		7	Died 1 hr. later in spite of glucose administration. •	33.1	11.2	5.7

Sokhey and Allan's results suggest that the increased excretion of nitrogen after insulin might be due to a breakdown of protein for the sake of glucose formation. This hypothesis would suggest that the production of urea should be increased in proportion to

the fall of blood sugar, and to the length of time the hypoglycemia is maintained. To test this hypothesis, in three experiments (Nos. 15, 16, 17, Table II) the blood sugar and the amino and urea nitrogen were followed in whole blood for 7 to 9 hours. In two of these experiments the urea not only showed no increase, but definitely decreased, contrary to the results obtained in the ten other experiments. These results indicate that the formation of urea is not dependent upon the level to which blood sugar falls, nor upon the duration of the hypoglycemia. The dosage of insulin likewise seems to have no influence on the amount of urea formed. The results suggest either that the low level of blood sugar is not itself the stimulus which causes the increased catabolism of nitrogenous material, or possibly that the nitrogenous reserve is limited in quantity, as otherwise prolonged hypoglycemia should cause a greater increase in urea. Milhorat and Chambers (1928) suggest that the extra nitrogen excreted after insulin has its origin in a reserve of "deposit protein" which may be depleted by fasting. In view of the recent work of Kiech and Luck (1928) it seems possible that this reserve may be merely the free amino acids of the tissues.

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ON THE CONFIGURATIONAL RELATIONSHIP OF 3-HYDROXYBUTYRIC AND 3-CHLOROBUTYRIC ACIDS.

WITH A FURTHER NOTE ON THE CONFIGURATIONAL RELATIONSHIP OF 3-HYDROXYBUTYRIC ACID AND METHYLPROPYL CARBINOL.

By P. A. LEVENE and H. L. HALLER.

(From the Laboratories of The Rockefeller Institute for Medical Research, New York.)

In recent years reports from several laboratories have appeared on the correlation of the configurations of hydroxy and of halogeno acids. The conclusions reached by different authors are quite contradictory. As an illustration, two pairs of acids may be mentioned; namely, lactic and chloropropionic acids and malic and bromo- or chlorosuccinic acids. According to Clough[1] and to Levene and Mikeska,[2] dextro-lactic acid is correlated with dextro-chloropropionic acid, whereas Freudenberg and coworkers[3] correlate it with levo-chloropropionic acid. In the succinic acid series, Clough,[1] Holmberg,[4] and Levene and Mikeska[2] correlate dextro-malic with levo-chloro- or bromosuccinic acid whereas Freudenberg[3] and Kuhn and Wagner-Jauregg[5] correlate it with dextro-bromosuccinic acid.

The disagreement seems particularly surprising inasmuch as Clough and Levene and Mikeska, on one hand, and Freudenberg, on the other, have used very similar methods. The cause of the disagreement lies in the fact that Freudenberg compares the

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[1] Clough, G. W., J. Chem. Soc., 113, 526 (1918).

[2] Levene, P. A., and Mikeska, L. A., J. Biol. Chem., 70, 365 (1926).

[3] Freudenberg, K., and Markert, L., Ber. chem. Ges., 60, 2447 (1927). Freudenberg, K., and Lux, A., Ber. chem. Ges., 61, 1083 (1928).

[4] Holmberg, B., Ber. chem. Ges., 61, 1893 (1928).

[5] Kuhn, R., and Wagner-Jauregg, T., Ber. chem. Ges., 61, 504 (1928).

behavior of the halogeno acids, not with that of lactic acid, but with the acyl derivatives of lactic acid, and follows the same path in correlating the substituted succinic acids. Levene and Mikeska, on the other hand, compared bromopropionic and bromosuccinic acids with the corresponding hydroxy acids. Freudenberg offers no rational argument in favor of his choice. The choice of Levene and Mikeska, however, is based on an earlier observation of Levene and Meyer.[6] From observations on hexonic acids, Levene postulated a rule which permits bringing out the allocation of the hydroxyl on carbon atom (2). When the difference between the numerical values of the rotations of the ion and of the undissociated acid has a plus sign, then the hydroxyl is attached to the right, and *vice versa*. However, when methylated gluconic acid was compared with methylated mannoic acid, the difference of the rotations between ion and undissociated acid was to the right in both cases, and hence it was concluded that hydroxy acids in which the hydrogen of the hydroxyl is substituted behave abnormally and that therefore such derivatives could not be used for comparison.

The arguments of Kuhn and Wagner-Jauregg[5] seemed to carry more weight. The method employed by these writers is fundamentally the same as the one which had been employed by Levene for correlating 2-aminohexonic acids with hexonic acids. Nevertheless, we must admit that all the methods thus far employed, including that of Kuhn, are indirect and that therefore conclusions reached by them are not infallible. Which one of the alternative views will have to be revised cannot be predicted. The present paper contains the suggestion of a method which may serve to correlate hydroxy and halogeno acids in a way more satisfactory than hitherto employed.

The fundamental assumption on which this method is based is the following: In aliphatic carbinols the substitution of the hydroxyl by a halogen proceeds without Walden inversion and leads to a halide rotating in the opposite direction from that of the carbinol. The reasons for this assumption were developed in the publications of Levene and Mikeska. Additional evidence may

[6] Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, **60**, 167 (1924); **65**, 535 (1925).

be found in the rotations of the derivatives of the optically active amyl alcohols and of other alcohols of the type[7]

$$
\begin{array}{ccc}
R_1 & & H \\
 & \diagdown \; \diagup & \\
 & C & \\
 & \diagup \; \diagdown & \\
R_2 & & CH_2OH
\end{array}
$$
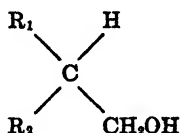
If this assumption is admitted, it is possible to correlate the configurations of the halogeno acids with the carbinols, and these have already been correlated with lactic acid. The process by which this task can be accomplished is seen from the following formulas.

		CH$_2$	CH$_2$	CH$_2$	
		|	||	|	
	COOH	CH$_2$	CH	CH	COOH
	|	|	|	|	|
COOH	CH$_2$	CH$_2$	CH$_2$	CH$_2$	CH$_2$
|	|	|	|	|	|
HCOH	HCOH	HCOH	HCOH	HCCl	→ HCCl
|	|	|	|	|	|
CH$_3$	CH$_3$	CH$_3$	CH$_3$	CH$_3$	CH$_3$
Levo.	Levo.	Levo.	Levo.	Dextro.	Dextro.
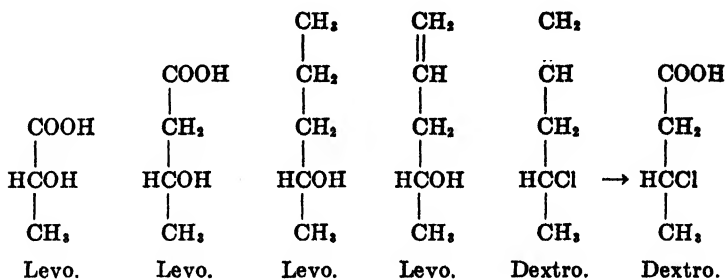
Thus on the basis of this set of reactions, dextro-3-chlorobutyric acid is correlated with levo-3-hydroxybutyric acid and hence with levo-lactic acid.

The same conclusion was reached previously by Levene and Mikeska[2] on the basis of the behavior of 3-hydroxybutyric and 3-chlorobutyric acids on passing from the ionized to the unionized state. In the pair levo-3-hydroxybutyric and dextro-3-chloro-butyric acids the difference $[M]_{ion} - [M]_{acid}$ has a minus sign.

It must be emphasized, however, that in a long series of hydroxy, halogen, thiol, and sulfo acids examined by Levene, Mori, and Mikeska[8] the butyric acids substituted in position (3) occupied an exceptional position. In these acids the thiol derivatives, on passing from the ionized to the unionized state, showed a change

[7] Unpublished results of Levene and Mikeska. ·

[8] Levene, P. A., Mori, T., and Mikeska, L. A., *J. Biol. Chem.*, **75**, 337 (1927). Levene, P. A., and Mori, T., *J. Biol. Chem.*, **78**, 1 (1928).

of rotation in the opposite direction from that observed in the sulfo derivatives, whereas in all other acids, the thiol derivatives, on passing from the ionized to the unionized state, suffered a change of rotation in the same direction as in the case of the sulfo acids. Hence, we do not venture to predict that in the other hydroxy and halogeno acids the conclusions reached by the older method will be supported by the newer method. We are now engaged in testing the older conclusions regarding the configurational relationships of hydroxy and of halogeno acids by the method described in this paper.

Incidentally, we have made use of the Δ^1-pentenol-(4), to test our earlier conclusion as to the configurational relationship of methylpropyl carbinol[9] to 3-hydroxybutyric acid. On the basis of our earlier work the following two substances should be configurationally related.

<pre>
      CH₃   CH₃
       | |
     HOCH   HOCH
       | |
      CH₂   CH₂
       | |
      CH₂   COOH
       |
      CH₃
     Levo.   Levo.
</pre>
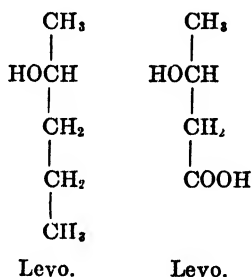
We have now confirmed this conclusion by the following set of reactions.

<pre>
      CH₃   CH₃   CH₃
       | | |
     HOCH   HOCH   HOCH
       | | |
      CH₂ ←   CH₂ →   CH₂
       | | |
      CH₂   CH   COOH
       | ‖
      CH₃   CH₂
     Levo.   Levo.   Levo.
</pre>
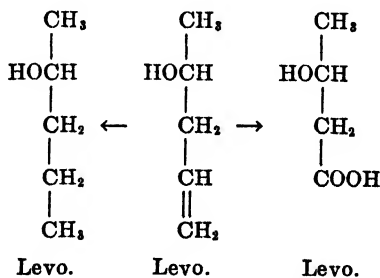
⁹ Levene, P. A., Haller, H. L., and Walti, A., *J. Biol. Chem.*, **72**, 591 (1927).

EXPERIMENTAL.

Δ^1-*Pentenol*-(4) (*Methylallyl Carbinol*).—The inactive carbinol was obtained on condensation of allyl bromide and acetaldehyde with zinc. The procedure for the preparation was the same as that described in preparing allylethyl carbinol.[10] The methylallyl carbinol boiled at 115–118°.

Resolution of Δ^1-*Pentenol*-(4) (*Methylallyl Carbinol*).—The acid phthalic ester of the carbinol was prepared by heating a solution of 50 gm. of carbinol and 86 gm. of phthalic anhydride in 100 cc. of dry pyridine on the steam bath for 1 hour. The acid ester was then isolated and purified in the same manner as described in the case of Δ^5-hexenol-(2).[11] It remained a thick syrup in spite of repeated attempts to crystallize it.

A warm acetone solution of the acid ester was treated with 1 equivalent of brucine. The solution was filtered and placed in the ice box; the brucine salt readily crystallized. It was recrystallized several times from acetone. This salt was decomposed with dilute hydrochloric acid and the phthalate extracted with ether; the ether extract was washed with water, dried over sodium sulfate, and the ether removed. The rotation of the phthalate in ether was

$$[\alpha]_D^{24} = \frac{+2.00° \times 100}{1 \times 25.0} = +8.0°.$$

From the mother liquors in the above resolution further crops of crystals were obtained. These on decomposition yielded a levo-rotatory phthalate whose $[\alpha]_D^{24}$ in ether was $-4°$. This acid phthalate was dissolved in a solution of sodium hydroxide (3 mols) and the solution was steam-distilled. The carbinol was extracted from the distillate with ether. The ether extract was dried over anhydrous potassium carbonate and the ether was removed. The carbinol, distilled at atmospheric pressure, boiled at 116–118.° In absolute alcohol it had the following rotation.

$$[\alpha]_D^{24} = \frac{-0.35° \times 100}{1 \times 9.4} = -3.7°.$$

[10] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **76**, 415 (1928).
[11] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **79**, 475 (1928).

The rotation in ether was

$$[\alpha]_D^{24} = \frac{-0.88° \times 100}{1 \times 16.6} = -5.3°.$$

Another lot of carbinol obtained on decomposition of the mother liquors in the resolution gave a carbinol whose rotation in a 1 dm. tube without solvent was −2.6°. In ether this carbinol (No. 1124) had the following rotation.

$$[\alpha]_D^{30} = \frac{-0.55° \times 100}{1 \times 8.94} = -6.1°.$$

α-Naphthylurethane of Levo-Δ¹-Pentenol-(4).—Levo-Δ^1-pentenol-(4) ($[\alpha]_D^{25} = -3.2°$ in ether) was converted into its α-naphthylurethane in the usual manner. Recrystallized from dilute alcohol, it melted at 47–49° and analyzed as follows:

0.1000 gm. substance: (Kjeldahl) 3.85 cc. 0.1 N HCl.
$C_{16}H_{17}O_2N$. Calculated. N 5.49.
    Found.   " 5.39.

In alcohol it had the following rotation.

$$[\alpha]_D^{30} = \frac{+0.28° \times 100}{2 \times 10.2} = +1.37°.$$

Dextro-Δ¹-4-Chloropentene.—45 gm. of levo-Δ^1-pentenol-(4), ($[\alpha]_D^{24} = -6.1°$ in ether, No. 1124) dissolved in 100 cc. of dry ether were slowly added to a suspension of 113 gm. of phosphorus pentachloride in 200 cc. of ether. The reaction mixture, kept cold by immersion in an ice water bath, was stirred with a mechanical stirrer. After the addition of all the carbinol, the bath was removed and stirring was continued until all the phosphorus pentachloride had dissolved, the temperature being maintained between 20–25°. The solution was then poured slowly into cracked ice and vigorously shaken. It was allowed to remain at room temperature for ½ hour with frequent shaking. The ether extract was washed successively with water, 2 per cent sodium hydroxide, and finally water. After drying over powdered calcium chloride for 24 hours, the ether was removed and the chloride was fractionally

distilled. A fraction which boiled at 95–97° was collected. The yield was 15 gm. It analyzed as follows:

0.1134 gm. substance: 0.1528 gm. AgCl.
C_4H_9Cl. Calculated. Cl 33.9.
Found. " 33.34.

In a 1 dm. tube without solvent $\alpha_D^{20} = +9.25°$. In ether the rotation was

$$[\alpha]_D^{20} = \frac{+1.00° \times 100}{1 \times 9.0} = +11.1°.$$

Dextro-3-Chlorobutyric Acid.—The chloride obtained in the experiment above was ozonized in chloroform in 5 gm. lots. A stream of ozonized oxygen was passed into the chloroform solution, which was immersed in an ice water bath, until a test portion no longer decolorized bromine in glacial acetic acid. The remaining chloroform was distilled off under reduced pressure and the ozonide poured into water. The mixture was vigorously shaken for ½ hour, warmed gently on the steam bath for 15 minutes, cooled, and then treated with a large excess of bromine. The mixture was vigorously shaken for 1 hour and allowed to stand overnight. The excess bromine was removed with a stream of air, the solution cooled in ice water, and treated with silver sulfate until free of bromine ion. It was then filtered and immediately treated with hydrogen sulfide to remove excess silver. The filtered solution was saturated with sodium sulfate and extracted with ether. The ether extract was dried over sodium sulfate and the ether removed. The residue was fractionally distilled. A fraction which boiled at 67–70°, p = 0.35 mm., was collected. It analyzed as follows:

0.1455 gm. substance: 11.25 cc. 0.1 N NaOH.
Theory for $C_4H_7O_2Cl$. 11.70.
0.0917 gm. substance: 0.1108 gm. AgCl.
Calculated. Cl 28.98.
Found. " 29.89.

In ether the substance had the following rotation.

$$[\alpha]_D^{20} = \frac{+1.20° \times 100}{1 \times 10.4} = +11.5°.$$

0.5645 gm. of the chloro acid was dissolved in 5.0 cc. of water and the rotation observed in a 1 dm. tube.

$$[\alpha]_D^{23} = \frac{+\ 2.43° \times 100}{1 \times 11.30} = +\ 21.5°; \ [M]_D = +\ 26\ 3°.$$

To 3.1 cc. of the above aqueous solution were added 3.0 cc. of 1.0 N NaOH. For the sodium salt,

$$[\alpha]_D^{23} = \frac{+\ 2.13° \times 100}{2 \times 6.77} = +\ 15.7°; \ [M]_D = +\ 22.6°.$$

Levo-Methylpropyl Carbinol (Pentanol-(2)).—The Δ^1-pentenol-(4) employed for reduction to the saturated carbinol was part of the lot used in the preparation of the dextro-Δ^1-4-chloropentene. 4 gm. of Δ^1-pentenol-(4) ($[\alpha]_D^{24} = -6°$ in ether, No. 1124) were dissolved in ether and reduced with hydrogen in the presence of colloidal palladium. The solution readily absorbed hydrogen and reduction was complete in 1 hour. The ether extract was dried over anhydrous potassium carbonate. After removal of the ether, the carbinol was distilled. It boiled at 118–120°. In a 1 dm. tube without solvent $\alpha_D^{20} = -6.50°$. In ether the rotation was

$$[\alpha]_D^{20} = \frac{-\ 1.10° \times 100}{1 \times 10.6} = -\ 10.4°.$$

It analyzed as follows:

4.215 mg. substance: 10.575 mg. CO_2 and 5 095 mg. H_2O.
$C_5H_{12}O$. Calculated. C 68 16, H 13 63.
       Found.   " 68 41, " 13 52.

α-Naphthylurethane of Levo-Methylpropyl Carbinol.[12]—The carbinol obtained in the foregoing experiment was converted into its α-naphthylurethane in the usual manner. Recrystallized from dilute alcohol, it melted at 78–80° and analyzed as follows:

0.1000 gm. substance: (Kjeldahl) 3.95 cc. 0.1 N HCl.
$C_{16}H_{19}O_2N$. Calculated. N 5 44.
       Found.   " 5 53.

[12] Levene, P. A., Haller, H. L., and Walti, A., *J. Biol. Chem.*, **72,** 591 (1927). Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **77,** 555 (1928).

In absolute alcohol the rotation was

$$[\alpha]_D^{23} = \frac{-0.87° \times 100}{2 \times 5.6} = -7.8°.$$

Levo-3-Hydroxybutyric Acid.—5 gm. of levo-Δ^1-pentenol-(4) ($[\alpha]_D^{25} = -3.2°$ in ether) dissolved in 40 cc. of chloroform was ozonized until a test portion no longer decolorized bromine in glacial acetic acid. The remaining chloroform was distilled off under reduced pressure and the ozonide poured into water. The solution was well shaken and 15 gm. of silver oxide were added. The mixture was heated under a reflux condenser on the steam bath for 1 hour, filtered hot, and the filtrate concentrated on the water pump to a small volume. The solution was again filtered and placed in the ice box. The silver salt readily crystallized. It was filtered off, washed with alcohol, followed by ether, and then dried. It analyzed as follows:

0.0864 gm. substance: 0.0440 mg. Ag.
$\quad\quad$ $C_4H_7O_3Ag$. Calculated. Ag. 51.11.
$\quad\quad\quad\quad\quad\quad$ Found. " 50 93.

0.2040 gm. of silver salt was dissolved in 5.0 cc. of water. The rotation of this solution was

$$[\alpha]_D^{20} = \frac{-0.14° \times 100}{2 \times 4.04} = -1.7°.$$

To 4.0 cc. of the above silver salt solution was added 0.8 cc. of 1.0 N HCl and the solution was filtered. For the free acid,

$$[\alpha]_D^{20} = \frac{-0.08° \times 100}{1 \times 1.58} = -5.0°.$$

0.4220 gm. of silver salt was dissolved in 5.0 cc. of water and 2 cc. of 1.0 N HCl were added. The solution was filtered and rotation observed immediately. For the free acid,

$$[\alpha]_D^{20} = \frac{-0.28° \times 100}{2 \times 3} = -4.66°.$$

THE DETERMINATION OF COPPER IN BIOLOGICAL
MATERIALS.*

By C. A. ELVEHJEM and C. W. LINDOW.

(*From the Department of Agricultural Chemistry, University of Wisconsin, Madison.*)

INTRODUCTION.

The wide distribution of copper in minute quantities in biological materials has been conceded for a long time. Its presence, however, in plant and animal life has been regarded, until very recently, as accidental.

Many early workers detected copper in certain plants, but attached no significance to its occurrence. Bucholz (1) and Meissner (2) in 1816 were probably the first definitely to establish the presence of copper in plant life. Sarzeau (3), in 1830, found copper in numerous plants, and since then many workers have reported the occurrence of this element, but most of them, as reviewed by Chevreul (4), have offered no explanation for its presence. More recently other investigators (5, 6) suggested that copper should be placed in the group of catalytic elements found in plants. Maquenne and Demoussey (7) in 1920 noted that copper was found in greater amounts in the more active organs of the plant; *e.g.*, young shoots and leaves. They concluded that copper in some way aided in the vital functions of the plant. McHargue (8) suggests that copper, together with other minor elements, plays an important rôle in life processes. Allison, Bryan, and Hunter (9) report that the additions of copper sulfate to the raw peat soils of the Florida Everglades stimulated the growth of a considerable number of agricultural plants. Whether this stimulation is due to the direct utilization of copper by the plant, which would indicate that copper was essential to plant growth, or to some indirect action of the element, remains to be determined.

The occurrence of copper in certain animal tissues was also recognized by early workers. Its function in marine organisms has been definitely established and has been reviewed by Rose and Bodansky (10). In the higher forms of animal life the rôle of copper has received little attention,

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although a few investigators, Bodansky (11), Guerithault (12), McHargue (13), have determined copper in animal tissues and supplemented their results with suggestions as to the functions of the metal.

The recent discovery, in this laboratory (14), of the importance of copper as a supplement to iron for hemoglobin building in the rat, definitely establishes the function of copper in the metabolism of at least one species of vertebrates, and points to the possibility that the element may have a similar function in other animals. This investigation has shown the necessity for an intensive study of the distribution of copper in nature. Before this end can be attained we must have a suitable method for the quantitative estimation of this element.

Survey of Methods.

Sarzeau (15), 1832, gives the first account of a quantitative method for the determination of copper in biological materials. This consisted essentially in removing the precipitate formed when ammonia was added to the solution of the ash, and precipitating the copper in the filtrate with potassium ferrocyanide from an acid solution   The precipitate was washed, dried, and weighed. Deschamps (16) in 1848 suggested precipitating copper with H_2S from an acid solution of the ash, dissolving the precipitate in HNO_3, adding alcoholic potash to form copper hydroxide, and weighing as CuO. Hill (17), 1902, was the first to determine copper colorimetrically by comparing the blue color formed by the addition of NH_4OH with that of a standard.

Guerithault (18), 1911, precipitated the copper from acid solution by means of H_2S, dissolved the CuS in acid, and collected the copper by electrolytic deposition.   Maquenne and Demoussey (19) employed a centrifugal method to remove the calcium and other insoluble sulfates, and subsequently separated the copper from the filtrate by electrolysis.   The deposit was dissolved, and the copper determined colorimetrically by use of potassium ferrocyanide.

Some of the more recent workers have used the well known xanthate method (20) for the estimation of copper.   The first determinations of copper, made in our laboratory, were by this method.   Samples such as liver, liver extract, lettuce, and cabbage were successfully analyzed according to this procedure.

The chief criticism of this method is that the solutions of copper xanthate are slightly turbid, and in order to obtain an accurate comparison the standard and the unknown solutions should contain identical amounts of copper per unit volume as Scott (20) suggests. This prevents the use of a colorimeter which we believe to be the most accurate method of making colorimetric comparisons. Other methods were investigated which would eliminate this difficulty.

The electrolytic method of Pregl (21) was tried but because of the large samples, which were often necessary, the concentration of other salts was so great that the complete deposition of the copper was not effected. Only 75 per cent of the total copper present in liver extract was recovered by electrolytic deposition, whereas complete recovery was obtained when a standard solution of copper sulfate was substituted for a sample. To separate the copper from the interfering salts before electrolysis would greatly increase the length of the procedure.

Redfield, Coolidge, and Shotts (22) report the satisfactory use of the micro method of Schoorl and Begemann with as little as 0.3 mg. of copper in the sample. They used this method for estimating copper in hemocyanin, which is relatively low in interfering elements and high in total copper. From 50 to 100 gm. of a dried food material would be required as a sample to give 0.3 mg. of copper, and therefore this method presents no advantages over earlier methods as far as the size of the sample is concerned.

Among several colorimetric methods which were investigated, that of Biazzo (23) was found to be the most satisfactory. This method is based upon the fact that the neutral solution, of a copper salt, when treated with a few drops of concentrated KCNS solution and a few drops of pyridine, gives a green precipitate of the composition $Cu(C_5H_5N)_2(CNS)_2$. The copper compound is soluble in chloroform and is removed quantitatively by the chloroform from the solution in which it was formed. Biazzo has shown that the green color of the chloroform layer varies in intensity according to its copper content. The method has a distinct advantage because it requires a small weight of sample. It is rapid, simple, and accurate as the recovery determinations on several plant and animal tissues show. This procedure, with such modifications as we have found necessary, is outlined as follows:

Method.

Samples varying in weight from 5 to 10 gm. are thoroughly ashed in an electric furnace at a temperature of dull redness until the carbon is completely destroyed. The ash is taken up in 15 cc. of HCl (1:1) and evaporated to dryness on the sand bath to render the silica insoluble. The residue is moistened with 5 cc. of 1 N HCl, after which 5 cc. of water are added. The sample is warmed on the sand bath for a half hour, filtered, and the insoluble residue washed thoroughly to a volume of about 100 cc. The filtrate is evaporated on a hot plate to about 10 cc. and, after cooling, transferred to a 25 cc. volumetric flask. Enough 1 N NaOH is added to make the solution just alkaline to phenolphthalein. Then 1 cc. of glacial acetic acid, 1 cc. of 10 per cent potassium thiocyanate solution, 10 drops of pyridine, and 5 cc. of chloroform, *accurately measured*, are added and the whole made up to volume with water. After thorough shaking, the chloroform layer is permitted to settle to the bottom of the flask, most of the aqueous layer is removed, and the remaining water and chloroform are transferred to a Bausch and Lomb colorimeter. The color is compared with that of a standard copper solution similarly treated.

Preparation of the Standard.—A $CuSO_4$ solution is prepared so as to contain 0.1 mg. of copper in 1 cc. of solution, by dissolving 0.3928 gm. of pure copper sulfate ($CuSO_4 \cdot 5H_2O$) in water and diluting to 1 liter. It is important that clear crystals of copper sulfate that show no sign of efflorescence be used. Then 0.5, 1, and 2 cc. of this standard solution are treated with acetic acid, potassium thiocyanate, pyridine, and chloroform in exactly the same manner as the sample solution and made up to 25 cc. The standard which has approximately the same intensity of color as the sample is selected, and the standard prism is usually set at 20 mm. When necessary the length of the standard column is varied.

Prevention of Contamination.—The greatest care must be observed to prevent the introduction of any copper from outside sources during the determination. Even reagents of the highest grade obtainable should not be used until found to be copper-free by actual test. The water used must be redistilled from glassware. The use of new porcelain dishes for the ignition of samples

is a source of copper contamination. All new porcelain dishes appear to contain appreciable amounts of copper, and these can be used only after having been properly treated.

The removal of the copper from the glaze of a porcelain dish is best accomplished by addition of an alcoholic solution of sodium acetate equivalent to 1 gm. of the salt, followed by ignition in an electric furnace, with subsequent extraction in 1:1 HCl for a period of several days. One thorough treatment of this kind eliminates the copper contamination from porcelain dishes. The importance of this precaution cannot be overemphasized since one 3 inch evaporating dish may contain as much as 0.033 mg. of copper. The quartz dishes used in this laboratory have been found to be free from copper, but even such dishes should be tested prior to their use.

Effect of Other Elements.—The effect of other elements on the development of the color has been tested by adding quantities of cobalt, nickel, manganese, and iron, 10 times greater than that found in an ordinary sample of biological material. The addition of 1 mg. of cobalt, nickel, manganese, or ferrous iron, separately or collectively, to a standard containing 0.1 mg. of copper, failed to influence the color development in the least. Ferric iron, however, when present in large amounts will give a brownish tinge to the chloroform layer, but out of a hundred food materials thus far analyzed this difficulty has been encountered in only a few cases. Animal tissues which are especially high in hemoglobin and commercial products made from them are probably the only samples which will give this trouble.

In such cases the copper must first be separated from the reduced iron by precipitation with hydrogen sulfide. This is accomplished by the following procedure: The ash is taken up in 10 cc. of 1:1 HCl, diluted, and filtered. The iron is reduced by adding a few granules of zinc, known to be free from copper, and boiled until the solution is colorless. Any undissolved zinc is filtered off, and the filtrate made to a volume of 200 cc., which gives a solution containing about 1 per cent of acid   The solution is warmed and H_2S is passed in for 15 minutes and the solution kept in a stoppered container until the CuS settles. The precipitate is filtered off on 7 cm. filter paper (Schleicher and Schüll No. 590). The filter paper containing the copper precip-

itate is thrown into 5 cc. of HNO₃ and the filter paper broken up. The acid solution is diluted and brought to boiling, and the disintegrated filter paper filtered off. The filtrate is evaporated to 10 cc., neutralized with 1 N NaOH, after which the original procedure is followed

We have used this same procedure for the determination of copper in iron salts with excellent results. A 1 gm. sample is generally used. If the compound is an organic salt, it must be ashed before being dissolved in the hydrochloric acid. A method suitable for iron salts is especially valuable at the present time when workers are interested in determining the amount of copper present in iron salts which are used in the treatment of anemia.

When the original method is applied to the determination of copper in milk, another difficulty is encountered. Because of the very small amount of copper in milk an unusually large sample is required. The large amount of calcium phosphate present in such a sample is precipitated upon neutralization of the solution and remains insoluble in the acetic acid solution. This voluminous precipitate prevents a quantitative extraction of the copper-pyridine-thiocyanate compound by the chloroform. In many cases no color whatsoever was obtained in the chloroform layer. Supplee and Bellis (24) suggested that the copper remains in the filtrate after two precipitations of the calcium phosphates with ammonia. This necessitates the use of large quantities of reagents and the subsequent evaporation of a filtrate of large volume. We have, therefore, applied the same procedure as that outlined for samples rich in iron, except that it is unnecessary to use zinc for the reduction of the iron. Recovery determinations have shown that H₂S will precipitate copper quantitatively from an acid solution of the milk ash. A 250 cc. sample is used for analysis. The ashing should be done in a quartz dish. This method may also be used for the determination of copper in other materials rich in calcium phosphate, such as bones.

Representative Results.

In Table I is given the copper content of three different materials as determined by the xanthate and Biazzo methods. It is shown that the results agree closely. The acidity, however, in the

xanthate method must be accurately regulated to obtain good readings.

As a check on the accuracy of the method, a record of the recovery of copper added to various samples is given in Table II.

TABLE I.

Comparison of Xanthate and Biazzo Methods.

Sample.	Cu per kilo dry matter.	
	Xanthate.	Biazzo.
	mg.	*mg.*
Liver, beef.....................................	66 0	71 0
" extract, Wilson Laboratories............	370 0	360.0
Lettuce..	11.0	10.3
Milk powder...................................	1.54	1.50

TABLE II.

Detailed Data Illustrating Procedure for Determination of Copper in a Few Representative Food Materials.

Sample.	Weight of sample.	Cu added.	Standard.		Unknown reading.	Cu	Recovery of Cu added.	Cu in sample. Dry basis.
			Cu	Reading.				
	gm.	*mg.*	*mg.*	*mm.*	*mm.*	*mg.*	*per cent*	*per cent*
Codfish (average)..............	5 0		0.1	20.0	13.4	0 1492		0.00298
"	5 0	0.1	0.2	20 0	16 1	0 2484	99 2	
Dates, dried (average).	10 0		0 05	20.0	18.8	0.0532		0.00053
" "	10.0	0.1	0.1	20 0	13.0	0.1538	100.6	
Lettuce, southern (average)...	5.0		0.05	20.0	17.2	0.0581		0.00116
" "	5 0	0.1	0.1	20 0	13 7	0.1460	87.9	
Oats (average)................	10 0		0.1	20 0	19.9	0.1005		0.00101
"	10 0	0.1	0.1	20 0	10.1	0.1980	97.5	
Plums, blue (average).........	10 0		0.1	20 0	20.7	0.0966		0.00097
" "	10 0	0.1	0.1	20.0	10.3	0.1942	97.6	

The determinations were made in triplicate, and 1 cc. of $CuSO_4$ solution equivalent to 0.1 mg. of Cu was added to the third sample prior to ashing. Although the size of the samples and their copper content varied considerably, the recovery of the copper was over 87.9 per cent in each case.

Table III gives the results of the analysis of a few representative iron salts. Most of the iron salts seem to contain copper in small quantities. Out of a total of ten samples only two were found to be copper-free. These figures are significant since they point to the possibility of copper playing a rôle in many cases when beneficial effects of iron salts in the treatment of anemia have been noted.

TABLE III.

Copper Content of Iron Salts.

Sample.	Cu per 1 gm. sample.
	mg.
Saccharated ferrous carbonate, I............................	0 0440
Ferrous carbonate...	None.
Ferric citrate...	0.0430
" ammonium citrate, I...............................	0 0204
" potassium tartrate................................	None.
Ferrous iodide syrup.	0.0083
Saccharated ferrous carbonate, II..........................	0 0192
" " " III.........................	0 0145
Ferric ammonium citrate, II..	0 0171
Saccharated ferrous carbonate, IV..........................	0 0182

TABLE IV.

Recovery of Added Copper in Analysis of Milk.

Sample No.	Volume of sample.	Cu present.	Cu added.	Total Cu found.	Recovery.
	cc.	*mg.*	*mg.*	*mg.*	*per cent*
I	250	0.0329	0.1000	0.1291	96 2
II	250	0 0409	0 1000	0.1365	95.6
III	250	0 0316	0 1000	0.1240	92.4

The analysis of three different samples of milk, together with the recovery of added copper, is given in Table IV. The recovery experiments show that it is possible to precipitate the copper completely by H_2S from an acid solution of the milk ash.

SUMMARY.

An accurate and rapid method, which is a modification of the original Biazzo method for the determination of copper in biological material, has been outlined.

Data are presented to show that samples containing as little as 0.02 mg. of copper can be analyzed with a high degree of accuracy.

Procedures for the analysis of substances rich in iron, for iron salts, and for milk and bones are given.

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STUDIES ON BLOOD CELL METABOLISM.

III. THE EFFECT OF METHYLENE BLUE ON THE OXYGEN CONSUMPTION OF THE EGGS OF THE SEA URCHIN AND STARFISH. THE MECHANISM OF THE ACTION OF METHYLENE BLUE ON LIVING CELLS.

By E. S. GUZMAN BARRON.

(*From the Laboratory of Research Medicine, Medical Clinic, the Johns Hopkins University, Baltimore, and the Marine Biological Laboratory, Woods Hole.*)

The use of methylene blue in the study of biological oxidations is based on the fundamental researches of Ehrlich (1). The dye seems, as Clark remarks "to have fallen into almost every conceivable use, ranging from an indicator in volumetric analysis to a therapeutic agent" (2). Because of the diversity of its applications, the action of methylene blue as a catalyst of biological oxidations has attracted great interest and the mechanism of these reactions, considerable discussion.

It is of interest at this point to examine briefly the changes in metabolism induced by addition of dyes to red blood cells. It was reported by Harrop and Barron (3) that methylene blue added to red blood cells causes an increased respiration, as shown not only by an augmentation of oxygen consumption but also by an increased CO_2 production. It was shown that the respiratory quotient of these cells after the addition of the dye is in the neighborhood of 0.76, indicating that the oxidation is complicated, affecting not only the carbohydrates, but the fats and proteins as well. In an effort to identify the nature of this action, the carbohydrate metabolism of red blood cells was studied by the same authors (4) with the result that methylene blue was shown to produce an indubitable increase in the carbohydrate oxidation. This was evidenced by a fall of the glycolytic quotient

$$\left(\frac{\text{Millimols lactic acid produced}}{2 \times \text{millimols glucose degraded}} \right)$$

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Further, it was shown that the oxidative action was not really peculiar to methylene blue but common to the series of reversible dyes possessing definite oxidation-reduction potentials with rH values in the vicinity of that of methylene blue.

Similar experiments with leucocytes, soon to be published, led to the belief that the catalytic action of methylene blue on respiration is not confined to blood corpuscles but is common to all living cells.   To test this hypothesis a series of experiments has now been performed to determine the effect of this dye on the respiration of sea urchin and starfish eggs.   Oxygen consumption alone was measured.

The experiments were performed in Warburg's micro respiration vessels with Barcroft's manometer, 0.2 cc. of M KOH being used to absorb the CO_2 produced.   The temperature of the bath was kept at $25° \pm 0.03°$.   The eggs which were collected in the middle of the summer of 1928, were received in flat bottomed dishes, and washed twice with sea water before use.   The methylene blue used was the same sample referred to in previous communications (purified methylene blue, zinc-free, from Leopold Cassella and Company, Frankfort-on-the-Main) and the solutions were made fresh every day in sea water.   The concentration in these experiments, as indicated below, varied from 0.001 to 0.005 per cent.

I. Action of Methylene Blue on Oxygen Consumption of Sea Urchin and Starfish Eggs.

Warburg's first communication (5) pointed out that unfertilized sea urchin eggs have a definite oxygen consumption which is augmented 8- or 10-fold on fertilization.   On the other hand, the oxygen consumption of starfish eggs does not rise appreciably after fertilization.

In experiments described in this paper the rate of oxygen consumption was first determined for a period of approximately 1 hour; the dye was then added and the oxygen consumption again determined.   About 5 minutes only were required to reach temperature equilibrium after addition of the dye as the room temperature was approximately equal to the thermostat temperature.

As can be seen from Fig. 1, which is an example of a series of similar experiments, the addition of methylene blue to these cells

definitely increases oxygen consumption. In all the experiments the increase was greater with starfish eggs than with sea urchin eggs. In general the observed increase in the case of sea urchin eggs was from 80 to 100 per cent of the normal rate, while with starfish eggs it was from 200 to 250 per cent. As was previously found for blood cells, the optimum concentration of methylene blue seems to be about 0.005 per cent. When the concentration is lowered to 0.001 per cent, the increase is considerably dimin-
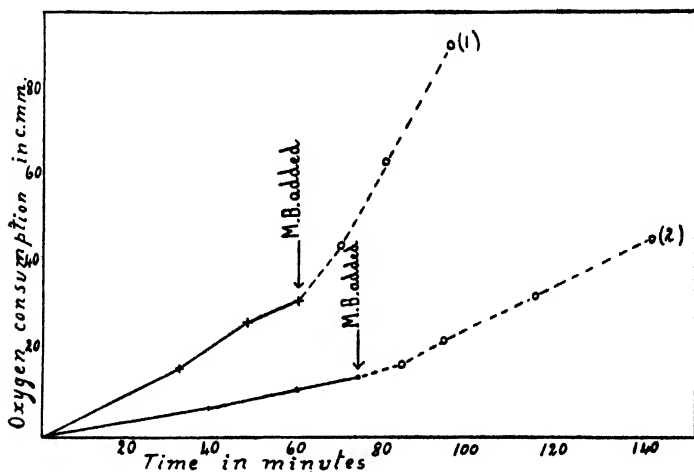
Fig. 1. The effect of methylene blue on the oxygen consumption of starfish and sea urchin eggs. Curve 1, oxygen consumption of starfish eggs; Curve 2, oxygen consumption of sea urchin eggs.

ished. As may be seen in Table I, there is a rough proportionality between the dye concentration and the increase of oxygen consumption, within the limits of dye concentration employed.

It is noteworthy that the action of methylene blue is not immediate. There is a more or less marked latent period which suggests the hypothesis that penetration of the dye through the cell membrane is necessary before its action can take place.[1]

[1] The fact maintained by Irwin (6) that chemically pure methylene blue does not penetrate the cell membrane does not interfere with this statement as not even the best preparation of methylene blue available is free from those supposedly less methylized dyes which do penetrate the cell wall.

II. Effect of Cyanides.

The mechanism of the inhibitory action of cyanides in cellular respiration is now well understood. We owe this knowledge to the splendid contributions of Warburg (7).   KCN stops the

TABLE I.

Influence of Methylene Blue Concentration.   Oxygen Consumption of Unfertilized Sea Urchin Eggs (Arbacia punctulata).

| Date. | O₂ consumption per 60 min. | | Concentration of methylene blue. | Increase of O₂ consumption after addition of methylene blue. |
	Before addition of methylene blue.	After addition of methylene blue.		
	c. mm.	*c. mm.*	*per cent*	*per cent*
July 24	11.0	24.2	0.005	120
" 25	18.6	30.0	0.002	61
" 26	15.0	20.0	0.001	33

TABLE II.

Action of Methylene Blue on the Oxygen Consumption of Starfish and Sea Urchin Eggs in Presence of 0.01 ʍ KCN.

O₂ consumption per 60 min.

Time, 60 min.	Before addition of KCN.	After addition of KCN.	After addition of methylene blue (0.005 per cent).
	c. mm.	*c. mm.*	*c. mm.*
Starfish eggs.			
I	38.5	0	20.0
II	41.0	0	24.2
III	40.0	0	23.1
Sea urchin eggs.			
I	14.4	0	14.0
II	13.3	0	13.2
III	10.0	0	10.4

respiratory process by forming a complex compound with the iron atom of the respiratory ferment which, according to Warburg's latest contributions (8), is a hemin compound in which iron is the reactive nucleus of hemin.   This inhibitory mechanism can be called a *specific action* as it is produced by direct action on the

catalyst. Warburg correctly designates KCN a "specific nega-
tive catalyst" (9).

Great interest has been stimulated by the striking observation
that methylene blue produces biological oxidations even in pres-
ence of cyanides and it has given a strong argument to the parti-
sans of Wieland's dehydrogenation theory (10) in their criticism
of Warburg's theory. It will be recalled that Thunberg (11) was
the first to show that succinic acid in absence of molecular oxygen
can be oxidized to fumaric acid by muscle enzymes in presence of
methylene blue and that the addition of cyanides has no influence
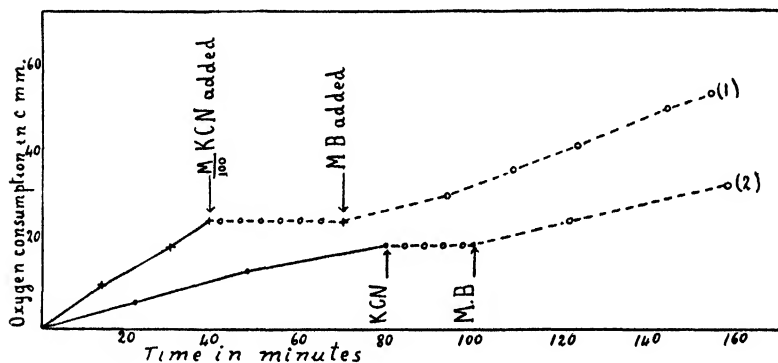
Fig. 2. The effect of methylene blue on the oxygen consumption of star-
fish and sea urchin eggs after addition of KCN (0.01 M). Curve 1, oxygen
consumption of starfish eggs; Curve 2, oxygen consumption of sea urchin
eggs.

on this reaction. Further Barron and Harrop (4) were able to
show that the action of methylene blue on red blood cells is not
altered by the addition of KCN and demonstrated that oxidation
of carbohydrates produced by methylene blue goes on in the pres-
ence of KCN.

In the present experimental series, after determination of the
normal rate of oxygen consumption of sea urchin and starfish eggs,
KCN was added in sufficient quantity (0.01 M) to produce a com-
plete stoppage of oxygen consumption. Half an hour or 1 hour
later methylene blue was added to the cells from the side arm of
the vessel and the oxygen consumption again determined for 1

hour or more.   As can be seen in Table II and Fig. 2, oxygen consumption was restored after the addition of the dye.   In the case of sea urchin eggs the oxygen consumption was practically restored to its normal level; this original level however was never reached in the case of starfish eggs, in which the value reached after addition of the dye was generally 50 per cent below the normal figure.   The amount of cyanide added is without influence upon the subsequent restorative power of methylene blue, so long as the cyanide concentration is strong enough to inhibit completely the normal oxygen consumption of the cells.   The same observation is reported by Fleisch (12) for the oxidation of succinic acid by muscle tissues in the presence of methylene blue and cyanides.   In view of the results it is interesting to note that, as shown by Perlzweig and Barron (13), sea urchin eggs have a high anaerobic metabolism demonstrated by the increased lactic acid production of the eggs after addition of KCN.

III. Effect of Narcotics.

That vital oxidations take place only when forces due to the particular structure of the cell, usually referred to as surface forces, are added to chemical forces is very well known.   Impairment of any of these forces brings inhibition of vital oxidations.   The restoration of cellular oxygen consumption by the addition of methylene blue after the normal specific chemical force   (Warburg's respiratory ferment?) has been inhibited by cyanides has just been described.   It was thought interesting to study the effect of methylene blue when the other factor, i.e. the general surface force, was abolished or its action lowered.

There are two means of destroying the general surface activity: (1) by mechanical means (cytolysis), (2) by addition of strongly adsorbable substances (narcotics).   Harrop and Barron have shown that the effect of methylene blue is greatly altered when the cell surface of avian erythrocytes is destroyed by repeated freezing and thawing, as in Warburg's experiments (14).   Thus addition of methylene blue to the bottom layer of hemolyzed goose red blood cells containing cell fragments and nuclei produces an increase of oxygen consumption of 21.6 per cent, while the increase is only 11.8 per cent on the top layer containing only a small amount of cellular debris.

It was therefore decided to use the second method of lowering the surface force in the experiments with marine egg cells. Meyerhof (15), working with sea urchin eggs (*Strongylocentrotus*), demonstrated at Naples that urethanes lower the rate of oxygen consumption when added to these cells, the inhibiting power being more effective the higher the member of a homologous series. Ethylurethane at different concentrations (3, 6, 8 per cent) and phenylurethane (saturated solutions) were employed as narcotics

TABLE III.

Effect of Narcotics. Action of Methylene Blue (0.005 Per Cent) on Oxygen Consumption of Sea Urchin and Starfish Eggs in Presence of Narcotics (Ethylurethane and Phenylurethane).

	Before addition of narcotics.	O₂ consumption per 60 min.		After addition of narcotics.	After addition of methylene blue.
		Narcotics used.			
	c. mm.			*c. mm.*	*c. mm*
Starfish eggs.					
I	25.4	Ethylurethane, 4 per cent.		14.7	21.8
II	27.0	" 4 " "		17.9	20.1
III	27.8	" 8 " "		12.2	17.4
IV	20.7	" 6 " "		12.5	16.8
V	20.5	" 8 " "		10.7	15.75
VI	16.9	Phenylurethane, concentrated.		5.6	12.0
Sea urchin eggs.					
I	13.7	Ethylurethane, 8 per cent.		9.1	7.3
II	18.0	" 3 " "		14 8	10.1

in the present experiments. The experiments were conducted in two ways: In the first series, after determining the normal rate of oxygen consumption of the cells (sea urchin and starfish eggs) the narcotics were added, and the oxygen consumption followed for approximately 1 hour. Methylene blue was finally poured into the main vessel from the side arm and the oxygen consumption determined. In another series of experiments, methylene blue was first added and then the narcotics.

After addition of urethanes, the oxygen consumption was

lowered in proportion to the concentration of narcotic, the inhibition being more marked, as was expected, with phenylurethane than with ethylurethane.   As can be seen in Table III, phenylurethane produced 67 per cent inhibition; ethylurethane however, even in such high concentrations as 8 per cent, produced only from 56 to 47 per cent inhibition.   In the case of starfish eggs,
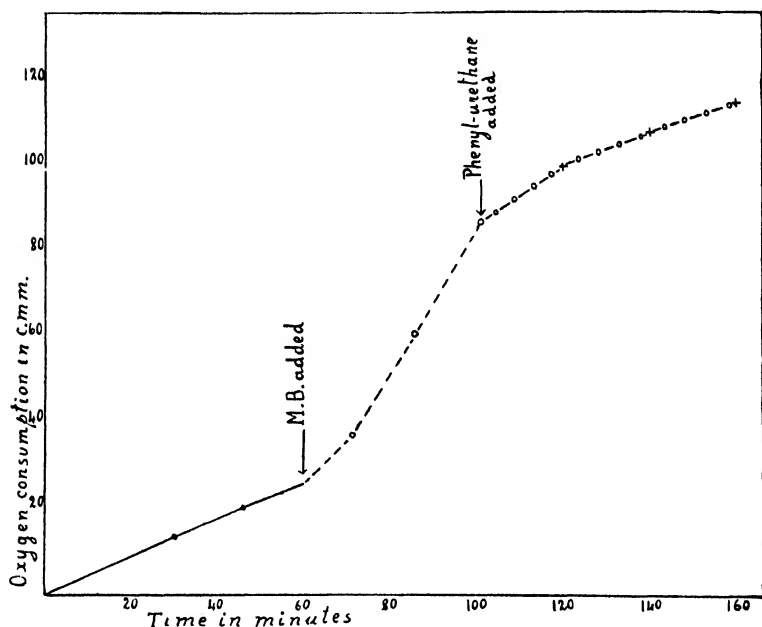
FIG. 3. The effect of narcotics on the oxygen consumption of starfish eggs. Showing the inhibitory effect of narcotics (phenylurethane) on the increased oxygen consumption previously produced by addition of methylene blue.

addition of methylene blue after narcotics increased somewhat (12 to 48 per cent) the oxygen consumption, but this never reached the level of normal eggs.   In the case of sea urchin eggs, addition of methylene blue after addition of narcotics produced an even lower oxygen consumption than after narcotic inhibition.   A striking result is obtained when oxygen consumption is first increased by addition of methylene blue and then the narcotic added

(Fig. 3). In such an experiment the effect of methylene blue is completely nullified and the cells respire at their normal rate.

Methylene blue, therefore, can be said to exert its catalytic effect as do the general enzymes. It acts at surface inter-
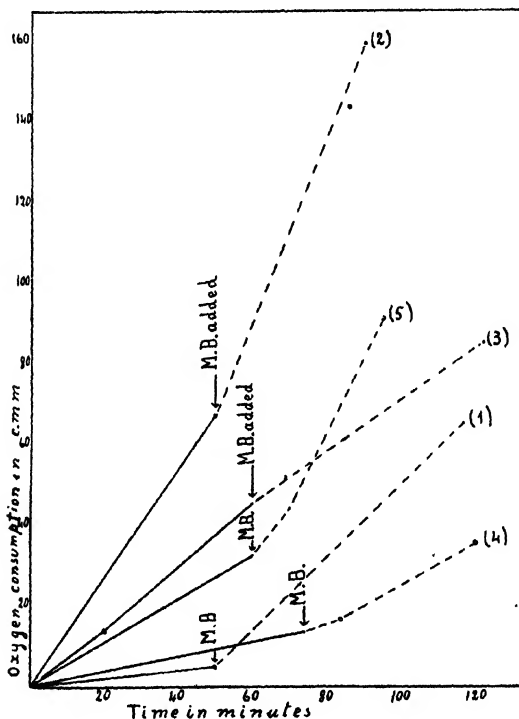
FIG. 4. The effect of methylene blue on the oxygen consumption of living cells. Curve 1, mammalian red blood cells; Curve 2, avian (goose) red blood cells; Curve 3, leucocytes (fresh lymph from the thoracic duct of dog); Curve 4, sea urchin eggs; Curve 5, starfish eggs. 'The solid line, before addition of methylene blue; the dash line, after addition of methylene blue.

faces, and impairment or destruction of these interfaces lowers or abolishes the activity of the dye.

Some years ago, Meyerhof (16) stated, as a result of his experiments on staphylococci, that though the respiration of living microorganisms is inhibited 10 to 40 per cent by methylene blue, the respiration of the same organisms, damaged or destroyed by acetone and heat, rises in the presence of methylene blue. He advanced the hypothesis that the dye may be able to replace a normal factor in an oxidizing mechanism when this factor has been inhibited or destroyed. The experiments reported elsewhere (3, 4) and those presented here do not confirm Meyerhof's hypothesis since the increased oxygen consumption produced by methylene blue has been observed on living cells, showing definite oxygen consumption previous to the addition of the dye.

Explanation of the mechanism of the oxidative action of methylene blue on living cells can best be made by recalling the essential facts to be drawn from the experiments of Harrop and Barron and those reported in this paper.

It has been established by experiments on cells with high anaerobic metabolism (mammalian red blood cells) and cells with high aerobic metabolism (avian red blood cells) that the action of methylene blue is stronger the higher the level of the anaerobic metabolism of the cell.   This can be clearly seen in Fig. 4 which shows graphically both the normal oxygen consumption and the methylene blue effect on (1) mammalian red blood cells, (2) avian red blood cells, (3) fresh leucocytes from the lymph of the dog's thoracic duct, (4) sea urchin and (5) starfish egg cells.   Furthermore, this action is not disturbed by total cyanide inhibition of the aerobic metabolism.   In addition to this it has been demonstrated (Barron and Harrop) that methylene blue effects its oxidative action on the carbohydrates of blood, only when these substances have been activated; i.e., transformed into more easily oxidizable compounds by the glycolytic ferment.   Thus inhibition of glycolysis in red blood cells either by destruction of the glycolytic enzyme (action of temperature) or by destruction of the cell surface (hemolysis in red blood cells possessing low glycolytic power) always stops the oxidative action of methylene blue.   This relationship appears more striking when the dye is added to chicken blood in which there is no glycolysis and the material incubated at 37° for some hours.   The action of methylene blue here is nil, as

the concentration of sugar and lactic acid remain unchanged. It can be concluded therefore that inhibition of the respiratory ferment by cyanide affects in no way the oxidative power of methylene blue, but that inhibition of the fermenting enzyme (glycolysis) prevents the dye action.

The increased oxygen consumption and the correlated increase of carbohydrate oxidation produced by methylene blue are in accordance with the well established fact that cells during respiration oxidize chiefly carbohydrates; methylene blue acts only during the first process of carbohydrate metabolism, *i.e.* during the anaerobic phase, its action starting when the carbohydrates have been "activated," or broken down into easily oxidizable compounds by the fermenting enzymes. As a reversible dye and a strong
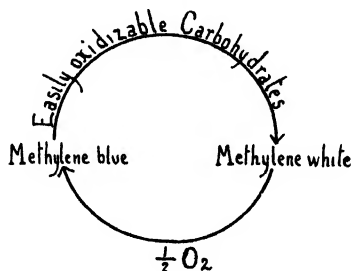
FIG. 5.

hydrogen acceptor, methylene blue accepts 1 H molecule from these compounds to be reduced to methylene white, which in turn is oxidized by the atmospheric oxygen. Thus there is established a cycle by which small amounts of the dye can carry on the oxidative process. This process can be expressed diagrammatically in the accompanying scheme (Fig. 5) and the action of methylene blue on living cells can be classified as a type of oxidative dehydrogenation, following in its mechanism the same principles as those expressed by Thunberg for the oxidation of succinic acid by muscle tissues in the presence of methylene blue, and based on Wieland's oxidation theory.

So much evidence has been accumulated during the last few years in support of Warburg's theory of the cellular respiratory mechanism that it can safely be assumed that iron-containing

compounds play the rôle of catalysts, even though it is not yet entirely clear whether iron activates the compounds to be oxidized or whether it activates molecular oxygen. From the experiments described above it is clear that oxidative dehydrogenations produced on living cells by addition of methylene blue can take their place along with the normal respiratory processes of the cells. Even though it is accepted that there is no relation between methylene blue oxidation and the normal respiratory process, there is a striking relationship between the fermenting enzymes and methylene blue action as the latter does not take place when the action of the enzyme is inhibited. It is possible, but not certain, that some of the oxidative processes of living cells are *normally* carried out by a mechanism equivalent to the methylene blue mechanism. Harvey's important contributions to the mechanism of bioluminescence (17) seem to prove that such oxidations can exist. He considers that the oxidation of luciferin to luciferase belongs to the type of oxidative dehydrogenations.[2]

<div align="center">CONCLUSIONS.</div>

1. The addition of methylene blue to living cells (sea urchin, starfish eggs) produces an increased oxygen consumption.

2. Cyanides added in sufficient concentration to produce complete inhibition of cellular respiration do not affect the oxygen consumption produced by methylene blue.

3. The addition of narcotics inhibits the action of methylene blue.

4. The increase of the oxygen consumption produced by methylene blue in a living cell is proportional to the level of its anaerobic metabolism. While marked in cells possessing high fermenting power (mammalian red blood cells), it is less marked or even nil in cells with low anaerobic metabolism (avian red blood cells, leucocytes).

5. The oxidative action of methylene blue on living cells belongs to the type of oxidative dehydrogenations; the dye plays

[2] Quastel's observations (18), Fleisch's experiments (12), and Stephenson's latest contribution (19) along the line of Thunberg's fundamental researches, also bring strong evidence to support the likelihood of normal oxidative dehydrogenations in the living cell.

its catalytic rôle on account of its reversibility and spontaneous oxidability by molecular oxygen without a catalyst.

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A TIME METHOD FOR DETERMINATION OF REDUCING SUGARS, AND ITS APPLICATION TO ANALYSIS OF BLOOD AND URINE.

By JAMES A. HAWKINS and DONALD D. VAN SLYKE.

(From the Hospital of The Rockefeller Institute for Medical Research, New York.)

During the development of a gasometric method (1928) for the determination of sugar we noticed that if an excess of sugar was added to the yellow ferricyanide reagent the resulting mixture during the heating became colorless. It has proved possible to use measurement of the time required for the decolorization of ferricyanide as a basis for estimating the sugar content of blood or urine. The method is so extremely simple and rapid that it serves to make sugar determinations possible when there is lack of either the time or the special apparatus required for colorimetric, gasometric, or micro titration procedures. The sugar estimation, although not so precise as the gasometric procedure, is sufficiently accurate for ordinary clinical purposes ($\pm$ 5 per cent of the sugar present), and requires only test-tubes and a beaker for apparatus. Since a half dozen determinations can be made simultaneously in 5 minutes, the procedure probably exceeds in rapidity any sugar analysis in current use. Oxalate and fluoride used as anticoagulants in blood do not interfere.

DETERMINATION OF REDUCING SUGAR IN BLOOD.

The blood sugar determination is made after removal of proteins by the Folin-Wu tungstic acid precipitant, and requires a volume of filtrate equivalent to 0.4 cc. of normal blood or 0.2 cc. of hyperglycemic blood.

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Reagents for Blood Sugar Determination.

Potassium Ferricyanide Reagent.—1 gm. of potassium ferricyanide, 75 gm. of anhydrous potassium carbonate, and 75 gm. of potassium bicarbonate are dissolved in water and made up to 1 liter.

The potassium carbonate and bicarbonate are dissolved in approximately 750 cc. of distilled water in a liter volumetric flask. The potassium ferricyanide is dissolved in about 100 cc. of distilled water and quantitatively added to the carbonate-bicarbonate solution. The mixture is made up exactly to a liter with distilled water and filtered. Filtration is necessary even though the solution appears perfectly clear. The solution is kept in a dark colored, glass-stoppered bottle. It will keep indefinitely in the dark. The reagents do not need to be weighed with any great degree of accuracy, except the potassium ferricyanide.

Tungstic Acid Solution.   (Mixed Reagents of Folin and Wu (1919).)

1 volume of 5 per cent sodium tungstate ($Na_2WO_4 \cdot 2H_2O$) is mixed with 1 volume of N/3 sulfuric acid. The acid solution is not permanent, but can be used for 2 weeks. A slight precipitate appears in a shorter time, but without diminishing significantly the effectiveness of the solution as a precipitant for the blood proteins. The solutions of N/3 sulfuric acid and 5 per cent sodium tungstate can be kept separately and mixed as needed.

Procedure for Blood Sugar Determination.

Precipitation of Blood Proteins.—A flask calibrated to contain 10 cc. is filled about two-thirds full with the tungstic acid solution, into which 2 cc. of blood are pipetted. Sufficient additional tungstic acid solution is added to make the volume up to the mark. An alternative procedure is to add the blood sample to 4 volumes of tungstic acid solution. The mixture is thoroughly shaken, and after 2 minutes or longer is filtered through a dry filter.

If the blood is known to be hyperglycemic only 1 cc. is taken, plus 5 cc. of tungstic acid, and is made up with water to 10 cc.

Decolorization of Ferricyanide by Blood Filtrate.—2 cc. of filtrate are pipetted into a Pyrex test-tube (14 by 125 mm. outside

measure[1]) followed by 2.00 cc. of ferricyanide solution.   Both solutions must be measured accurately from Ostwald bulb pipettes with capillary stems.   The ferricyanide should be added last, in order to facilitate its mixture with the lighter filtrate.   The tube is shaken to mix the two solutions, and is then immersed in a beaker of water which is boiling gently.   A series of tubes may be heated at once; it is convenient to use a cylindrical copper rack, such as is employed by bacteriologists, to hold the tubes.[2]   A tube containing distilled water is immersed with the other tubes to facilitate by comparison the detection of the moment when the ferricyanide solutions are decolored.   Also the tubes should be observed against a white background, formed by a white wall, or by white paper or adhesive tape attached to the side of the beaker away from the observer.   The time in seconds for each tube is taken, preferably with a stop-watch, from the moment the tube is immersed in the boiling water until the last trace of yellow disappears.

With the prescribed amount of ferricyanide and size of test-tubes, 50 mg. of glucose per 100 cc. of blood decolorize the reagent in 390 seconds, and 300 mg. of glucose per 100 cc. of blood will decolorize it in 88 seconds, when the blood filtrate represents a 1:5 dilution of the blood.   One can work between these time limits.   However, if the reagent is decolorized in less than 120 seconds (equivalent to more than 170 mg. per cent of blood sugar) it is preferable to dilute another portion of blood filtrate with an equal volume of water and repeat the analysis with the diluted filtrate in order to obtain a longer decolorizing period and more exact results.

Graphic Calculation of Results of Blood Analysis.

The number of milligrams of sugar per 100 cc. of blood is read directly from the curve shown in Fig. 1 when the 2 cc. of filtrate used represent 0.4 cc. of blood.   When the filtrate is twice as dilute (hyperglycemic blood) the blood sugar content indicated by the curve is doubled.

[1] If tubes of different dimensions are used than those specified it is necessary to make a new curve by determining the length of time required by standard glucose solutions to decolorize the reagent.

[2] These copper racks may be obtained from Eimer and Amend, New York, Catalogue No. 32002, or from Arthur H. Thomas Company, Philadelphia, Catalogue No. 9488.

DETERMINATION OF REDUCING SUGAR IN URINE.

The procedure here outlined is designed for use with urines such as those encountered in diabetes, in which the significant variations in glycosuria are gross enough to be satisfactorily shown by measurement of the total reducing substances. The method is accurate to within 0.1 per cent of glucose in urine. Unusually concentrated normal urines may have reducing substances equivalent in reducing power to as much as 0.4 per cent glucose. The reducing substances are almost entirely non-fermentable (Eagle, 1926–27; Hawkins, MacKay, and Van Slyke, 1928), and have no apparent relationship to carbohydrate metabolism. In view of these facts the technique was regulated to determine reducing substances in concentrated urine in concentrations of 0.5 per cent and above, and in dilute urine in concentrations of 0.25 per cent and above.

Reagents for Urine Sugar.

Ferricyanide Solution.—Same as for blood sugar.

Procedure for Urine Sugar.

Dilution of Urine.—Ordinarily 1 cc. of urine is diluted with water to 50 cc. In urine so diluted the ferricyanide reagent used will determine up to 3 per cent of glucose. In case the sugar content is known to be above 2 per cent 1 cc. of urine is diluted to 100 or 200 cc., so that glucose up to 6 and 12 per cent respectively can be determined. On the other hand, if the urine as voided is obviously dilute and presumably of low sugar content, it is well to dilute only 25-fold. Dilution is the only preliminary treatment of the urine required. Even albumin does not affect the determination significantly.

Decolorization of Ferricyanide by Diluted Urine.—2 cc. of diluted urine are pipetted into a Pyrex test-tube (14 by 125 mm. outside diameter) followed by 2.0 cc. of ferricyanide solution. The procedure from this point is exactly the same as in the blood sugar method described above.

The amount of sugar in the urine is found by use of the curve in Fig. 1. If the urine has been diluted 100-fold instead of 50-fold, the sugar concentrations indicated by the curve are to be doubled.
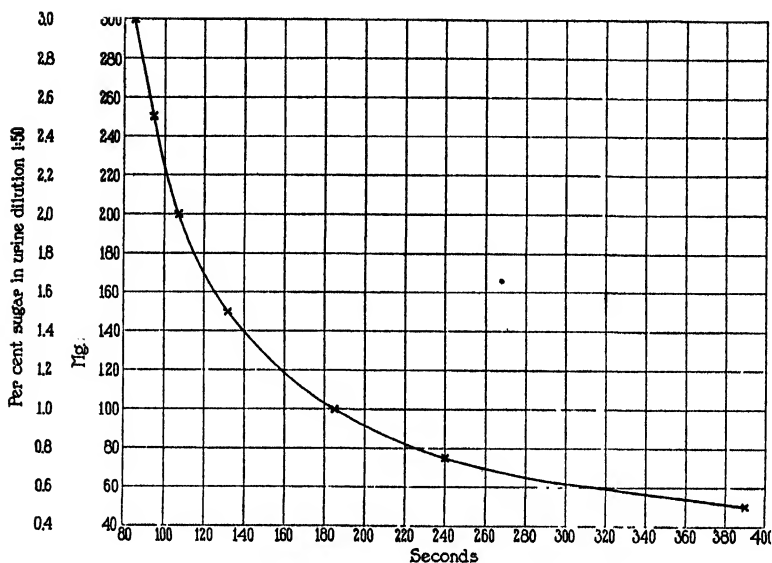
FIG. 1. Time curve of reduction of ferricyanide by standard glucose solutions under conditions of blood and urine determinations. Abscissæ represent time in seconds required by glucose standards to decolorize ferricyanide reagent. Each cross represents the average of several determinations with glucose standards. Ordinates represent per cent sugar in urine when urine is diluted 1:50, or mg. of sugar per 100 cc. of blood when blood is diluted 1:5. If other dilutions are used, sugar contents represented by the ordinates are multiplied or divided accordingly. *E.g.* if urine dilution is 1:25 instead of 1:50, multiply by 0.5 the sugar content indicated by the curve.

TABLE I.

Determination of Time Required by Various Concentrations of Glucose to Decolorize Ferricyanide Reagent under Conditions of Blood Determination.

Glucose concentration in standard solution, *mg. per cc.*.........	0 6	0.5	0.4	0.3	0.2	0.15	0.1
Glucose represented in portion of solution used for determination, *mg.*.............................	1.2	1.0	0.8	0.6	0.4	0.3	0.2
Time required to remove color from reagent, *sec.*..............	89	96	107	132	187	236	392
	88	96	108	134	185	244	390
	88	96	109	130	183	240	390
Average..........................	88	96	108	132	185	240	390

EXPERIMENTAL.

In order to determine the rate at which glucose reduces ferricyanide, the reagent was heated with standard glucose solutions
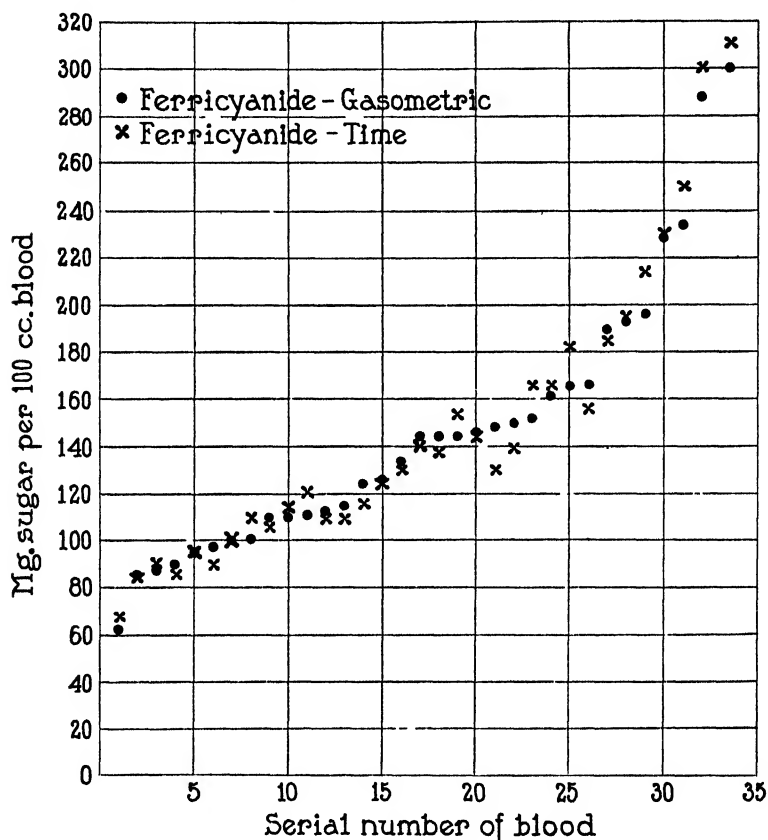
Fig. 2. Comparison of sugar found in blood by the Van Slyke-Hawkins gasometric and the time methods. Ordinates represent mg. of sugar per 100 cc. of blood. Each symbol represents the average of duplicate determinations.

of various concentrations under the conditions described above for blood and urine analysis. 1 volume of reagent was mixed with 1 volume of glucose solution.

The results are shown in Fig. 1, and Table I. They indicate that a reproducible curve is obtained by means of which it is possible to determine the amount of glucose in a solution from the time taken to decolorize the ferricyanide reagent.

Comparison with Gasometric Blood Sugar Method.

Comparison of results obtained by the Van Slyke-Hawkins gasometric method with those yielded by the present blood sugar

TABLE II.

Recovery of Glucose Added to Blood.

Blood No.	Mixture analyzed.		Sugar found.	Observed increase due to added glucose. (a)	Calculated increase due to added glucose. (b)	Observed increase minus calculated increase. (a) − (b)
	Blood.	Glucose added.				
	cc.	*mg.*	*mg. per 100 cc. blood*	*mg. per 100 cc. blood*	*mg. per 100 cc. blood*	*mg. per 100 cc. blood*
1	5	0	90		0	
	50	100	280	190	200	−10
	20	20	190	100	100	0
	20	10	140	50	50	0
	20	5	110	20	25	−5
2	5	0	95		0	
	50	150	400	305	300	+5
	50	100	300	205	200	+5
	50	50	192	97	100	−3
3	5*	0	26*		0	
	20	40	230	204	200	+4
	20	20	125	99	100	−1

* Fermentable sugar had been removed by spontaneous glycolysis, residual sugar found by gasometric method.

method in analyses of thirty-three bloods, normal and pathological, is shown in Fig. 2. The two methods agreed, usually within a few mg. per 100 cc., except where the sugar concentration was above 200 mg. per 100 cc. of blood. In those results for high sugar values, which did not agree quite so well, the time periods fell on the steep part of the curve, where ferricyanide is reduced rapidly. In all these analyses the same dilution of blood filtrate, from blood + 4 volumes

of precipitant, was used.   More accurate results can be obtained
with blood of high sugar content if, as recommended above, the
filtrate represents a 10-fold instead of a 5-fold dilution of the blood.

TABLE III.

*Recovery of Glucose Added to Urine.   Comparison of Time and Gasometric
Methods.*

Urine No.	Albumin in urine.	Glucose added.	Sugar found.		Increase found due to added glucose. Time method.	Error. Time method.
			Gasometric.	Time method.		
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1	0	0	0.4			
1	0	1.0	1.4	1.5	1.1	+0.1
1	0	˙0.5	1.0	1.0	0.5	+0.1
1	0	0.30	0.7	0.8	0.5	+0.1
2	0	0	0.18			
2	0	2.0	2.18	2.2	2.0	0.0
2	0	1.0	1.18	1.2	1.0	0.0
2	0	0.5	0.68	0.7	0.5	0.0
2	0	0.25	0.43	0.4	0.2	−0.1
3	1.8	0	0.3		˙	
3		4 0		4 2	3.9	−0.1
3		2.0		2.2	1.9	−0.1
3		1.0	1.4	1.3	1.0	0.0
3		0.5		0.8	0.5	0.0
3		0.25		0.5	0.2	−0.05
4	1.0	0	0.2			
4		4.0		3.9	3.7	−0.3
4		2.0		2.2	2.0	−0.0
4		1.0	1.3	1.1	0.9	−0.1
4		0.5		0.6	0.4	−0.1
4		0.25		0.4	0.2	−0.05
5	0	0	3.75	3.6		

Recovery of Glucose Added to Blood.

Tests were made to see whether glucose added to blood was
recovered.   The results, given in Table II, show that all the added
sugar is recovered.

Recovery of Glucose Added to Normal and Albuminous Urine, and Comparison with the Gasometric Urine Method.

In Table III are given the results of analyses of normal and albuminous urines to which known amounts of glucose were added and the comparison with the Van Slyke-Hawkins method. The two methods agree within 0.1 gram of sugar per 100 cc. of urine when the urine is diluted 1:50 or 1:100, and within 0.2 gram when the urine is diluted 1:200. The added glucose is recovered within the limits of error of the method.

SUMMARY.

Reducing sugars are determined by the rate at which they reduce yellow ferricyanide to colorless ferrocyanide. The amount of sugar is measured by the time required (100 to 300 seconds) for disappearance of the yellow color.

Applications to rapid determination of sugar in blood and urine are described.

The accuracy of the method, ± 5 per cent, is less than that of the gasometric ferricyanide procedure previously published (Van Slyke and Hawkins, 1928). Extreme speed and simplicity of operation, however, and the obviation of special apparatus make the present method desirable when rapidity is essential, or when simplicity in equipment and procedure are important.

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MUSCLE PHOSPHORUS.

II. THE ACID HYDROLYSIS OF LACTACIDOGEN.*

BY H. A. DAVENPORT AND JACOB SACKS.

(From the Institute of Neurology and the Department of Physiology, North-western University Medical School, Chicago.)

Although the identities of all of the compounds of phosphorus present in an acid extract of muscular tissue are not yet known, it is possible to obtain in mammals at least five reasonably definite end-points of different fractions. This separation is based upon the ease of conversion of the phosphorus contained in the compounds to orthophosphate by acid and by enzyme.

The values obtainable for the inorganic fraction, which is made diffusible by an acid protein precipitant, are sharply defined (14). This free phosphate, when precipitated by magnesia mixture from an unhydrolyzed extract of resting mammalian muscle is usually less than 20 mg. per cent. When the same extract is hydrolyzed at room temperature by means of acid molybdate (Briggs' reagent) a second sharply defined value is obtained. This one is well known as Embden's A value, and represents the sum of the inorganic phosphate (I value) and the organic fraction which has been identified as phosphocreatine (7, 13).

If one has put the sample of finely divided muscle into a 2 per cent solution of sodium bicarbonate and incubated at 37–40°, a third phosphorus value is obtained. This was called by Embden the B value, and the amount represented by B−A was considered to be that freed by the enzymatic hydrolysis of a carbohydrate ester (4–6). Lohmann (12) has questioned the origin of this fraction and stated that it was pyrophosphate instead of a hexosephosphoric acid ester. Also (11) he has repeated the work of Embden by isolating hexosephosphate from muscle, and

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* The first paper of this series is cited under reference 14.

subjected the purified material to acid hydrolysis (1 N HCl in a boiling water bath). A constant for a first order reaction was not obtained, hence he concluded that the material was a mixture of hexose esters.

The work reported here, in which it will be shown that Embden's lactacidogen[1] is separable into two components, resulted from attempts to find a method for determining the B value without resorting to enzymatic hydrolysis. We suspected that it might be possible to separate into two or more portions the fraction which is included between the A value and the total. This fraction in rabbit gastrocnemii is about 80 mg. per cent, and includes both the lactacidogen phosphorus and an enzyme-stable portion.

Since the question of diffusibility of phosphorus-containing compounds has been raised by Irving and Wells (10), we made some preliminary experiments to determine whether the total acid-soluble phosphorus could be extracted in as short a time as 15 minutes. After adding the thinly sliced frozen muscle to the trichloroacetic acid solution and weighing, the flask was shaken vigorously for 15 minutes and about half of the fluid filtered off. The remainder was allowed to remain with the tissue in a refrigerator overnight and determinations of total phosphorus by wet ashing were made on the two portions of filtrate. The amount of phosphorus found in the filtrate which had remained the longer time with the tissue, was only about 5 per cent more than that in the portion removed at the end of 15 minutes. Hence we have used the rapid method of extraction. It is true that the A value increases in acid filtrates on standing for several days at room temperature, but this appears to be due to the hydrolysis of the material in the lactacidogen fraction rather than to a phenomenon of equilibrium between tissue and fluid.

The results obtained by hydrolyzing a fresh trichloroacetic acid extract of muscle in 1 N mineral acid in a boiling water bath showed the phosphorus-containing material included between the A value and the total to be separable into two fractions. This separation could be based upon a definite change (which occurred near the end of the 1st hour) in the velocity of hydrolysis. We

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[1] The term "lactacidogen" has been used in its originally established terminology and does not refer specifically to hexosephosphate. See Embden, G., and Jost, H., *Z. physiol. Chem.*, **179**, 24 (1928).

have referred to the value obtained for phosphorus liberated after 1 hour as the H value to distinguish it from Embden's B value.

Procedure.

The gastrocnemii of rabbits were used as the source of muscular tissue. The animals were prepared as follows: A sedative dose of amytal (30 mg. per kilo) was administered intraperitoneally, and when the rabbits became somnolent the lumbar region of the spinal cord was injected with 0.2 to 0.5 cc. of 95 per cent ethyl alcohol. Complete surgical anesthesia of the hind legs was obtained and the dose of amytal used was not large enough to produce complete anesthesia or cause slowing of respiration. The muscles were dissected free from surrounding structures but care was taken not to disturb the blood supply. Their origins and insertions were left intact also. Such a preparation provided well oxygenated muscles whose motor innervations had been blocked in the cord and whose peripheral nerves were available for stimulation.

In each instance the resting and stimulated gastrocnemii used for direct comparison of analytical data were taken from the same animal. Faradic stimulation of 30 to 45 seconds duration through the motor nerve was used. The secondary of the inductorium was set to give a stimulus that was just maximal and the frequency of interruption of the primary circuit was about 8 per second. The stimulated muscle was frozen *in situ,* and freezing begun after about 15 seconds of stimulation and completed as nearly as possible during the succeeding 30 seconds. Stimulation was continued until freezing of the nerve occurred or until the muscle was frozen completely.

The method of freezing and of preparing the muscle samples for analysis has been described in previous reports (2, 14).

In order to make accurate comparisons between incubated and unincubated samples it was necessary to obtain representative samples of each. This was accomplished by putting successively every third cross-section of the muscle into 2 per cent sodium bicarbonate solution while the other two were put into 5 per cent trichloroacetic acid. Valid comparisons could then be made between the B value obtained by incubation and the hydrolytic (H) value obtained by acid.

Fractional acid hydrolyses were made on 20 cc. of muscle fil-

trate to which were added 20 cc. of water and 10 cc. of 5 N sulfuric
acid.   Samples of 5 cc. were withdrawn at time intervals shown
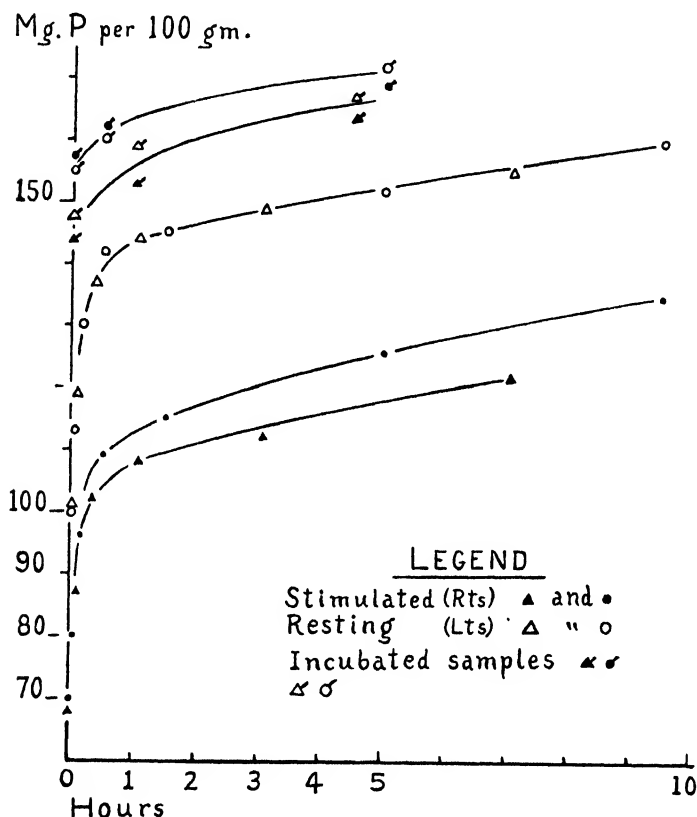
FIG. 1. The rate of acid hydrolysis of muscle filtrates obtained from
incubated and unincubated portions of rabbit gastrocnemii.   Points on the
chart indicated by circles are from data obtained from samples taken from
the right (Rts.) and left (Lts.) legs of one animal, and those indicated by
triangles represent data from those of another.   Note that the start for
the hydrolysis of the incubated samples represents the B values and that
the stimulated muscle agrees approximately with its unstimulated homo-
logue only by enzyme hydrolysis, not by acid.

on Fig. 1.   The samples were discharged into graduated tubes
which contained 25 cc. of water at ice bath temperature.   This

solution was made up to a final volume of 40 cc. and phosphorus determined by the procedure of Fiske and Subbarow (8).

Total acid-soluble phosphorus was determined by wet ashing an aliquot of the original filtrate and estimating colorimetrically as above.

A glance at Fig. 1 shows that the substance or substances which yield phosphate on acid hydrolysis break down with great rapidity during the first few minutes and that after about 1 hour the rate of reaction simulates a linear function. Such a condition would be furnished by a mixture of two substances, one rapidly hydrolyzable and the other very slowly hydrolyzable. However, each component might be a mixture of two or more substances. Since the break in the curve occurs at the end of about half an hour, it appeared that most of the substance or substances undergoing rapid hydrolysis would be hydrolyzed in 1 hour. Hence we have used 1 hour as the arbitrary length of time in determining our H values.

The shape of the curve shows that one is able to determine a value for one of the phosphorus-containing fractions in muscle, which has as definite an end-point as the Embden B value and which appears to have just as much physiologic significance.

The values for total phosphorus show that it would require a very much prolonged hydrolysis for the difficultly hydrolyzable portion to reach its end-point when acted upon by 1 N acid.

If one compares the form of the curve obtained from the acid hydrolysis of a fresh muscle filtrate with that obtained by Lohmann ((11) p. 315) for the hydrolysis of Embden's ester, it will be noted that it required 10 hours to produce a 40 per cent hydrolysis of Lohmann's purified material, whereas the whole of a fraction corresponding approximately in quantity to that designated by Embden as lactacidogen (B − A) is hydrolyzed (in an extract of resting muscle) in about an hour. The material isolated by Lohmann conforms in rate of hydrolysis to the difficultly hydrolyzable fraction (T − H)—not the lactacidogen. Since the portion of phosphate freed by acid at boiling water bath temperature might be an entirely different fraction from that liberated by enzymatic hydrolysis, experiments were made to decide its identity.

The protein of the incubated samples was precipitated by adding

a volume (15 cc.) of 10 per cent trichloroacetic acid equal to the volume of the bicarbonate solution.   The protein-free filtrate was subjected to hydrolysis by 1 N acid in the same manner as the unincubated samples.   The rate of increase of free phosphate in the hydrolysate of the incubated muscle was found to be approximately the same as that in the unincubated hydrolysate after the 1st hour of rapid hydrolysis of the latter (upper two curves in Fig. 1).   This indicated that the phosphate freed during the 1st hour of acid hydrolysis of unincubated resting muscle was contained in that portion hydrolyzed by the muscle enzymes.

The portion of orthophosphate freed by 1 hour's acid hydrolysis agrees in quantity with that designated by Lohmann (12) as originating in pyrophosphate.   We have attempted to confirm this observation.   Under the conditions of experiment it was found that mixtures of ortho- and pyrophosphate, in quantities comparable to that expected in a muscle filtrate, when subjected to acid hydrolysis, would give a rate of conversion of the pyrophosphate to orthophosphate which agreed approximately with the rate of conversion of the material in a fresh filtrate.   It was found also that pyrophosphate gave a blue color with Fiske's reagents. The color formed at room temperature but required nearly an hour to begin to appear.   It had a characteristic ultramarine tint which differed markedly from the gray-blue produced by orthophosphate.   It was considered that the color might be produced by a conversion of pyro- into orthophosphate, but the marked difference in tint indicated rather that a colored complex was formed slowly by the pyrophosphate itself.[2]   We applied this color test to fresh trichloroacetic acid muscle filtrates to which pure sodium pyrophosphate had been added, and found that these mixtures developed color in a different manner than filtrates to which no addition had been made.   The quantities of pyrophosphate added (0.02 to 0.06 mg. of P in 25 cc. total volume) varied from equivalents of 10 to 30 mg. per cent of added phosphorus when calculated on the basis of the size of muscle sample.   The effect of the pyrophosphate became readable in a colorimetric comparison after about $\frac{1}{2}$ hour and the pyro color became so marked

[2] Mellor, J. W., (A comprehensive treatise on inorganic and theoretical chemistry, London, 1928, 8, 976) states that W. Gibbs has obtained complexes of pyrophosphoric and tungstic acids.

after 2 hours that it could not be matched accurately against the color produced by muscle filtrate alone.

The tests were checked against an orthophosphate standard, and against comparable mixtures of ortho- and pyrophosphate. Although muscle filtrates alone showed a slight increase in color during the 2 hours they were observed, the characteristic tint
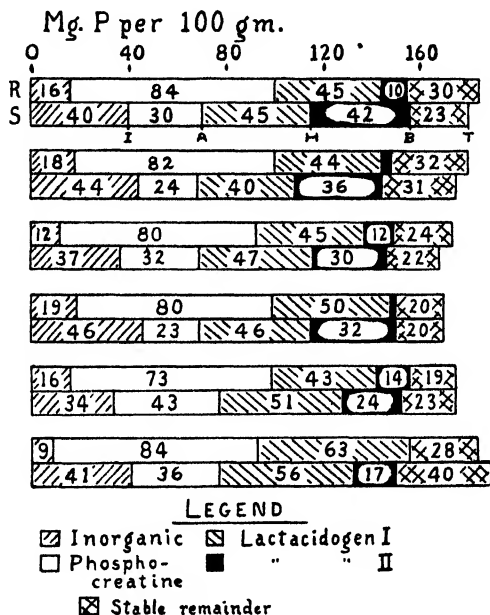
FIG. 2. The distribution of acid-soluble phosphorus in the gastrocnemii of six rabbits. *R*, resting; *S*, stimulated (30 to 45 seconds indirect tetanization). The pair of muscles from each animal is charted adjacently for ease of comparison.

of pyrophosphate was not seen in them. Samples to which pyrophosphate was added could be matched for tint as well as intensity provided a comparable mixture of ortho- and pyrophosphate were used for the standard. Hence the color tests failed to confirm Lohmann's preliminary report that there is about 40 mg. per cent of phosphorus as pyrophosphate in rabbit muscle. It was found impossible to demonstrate the presence of any free pyro-

phosphate in fresh muscle filtrate unless it were added, but an amount equivalent to 10 mg. per cent of phosphorus could be detected readily when added.   There remains the possibility that pyrophosphate might exist in combination with an organic radicle, and further work will be necessary to settle the point.

Fig. 2 shows changes in the distribution of acid-soluble phosphorus which occur during brief stimulation, when recovery changes are minimized by freezing the muscle during stimulation. There is a marked rise in I, a fall in A and H, but only a slight fall or no change in B.   The charting as the fourth fraction, of the portion which is enzyme-labile but acid-stable (B − H or Lactacidogen II), is based upon the chemical methods by which it has been demonstrated rather than a physiologic basis.   It is conceivable that this fraction rightly represents some transfer of phosphate from the phosphocreatine, because the acid-labile material (H − A), Lactacidogen I, undergoes no significant change in quantity during brief stimulation.

Andrews (1) found that stimulation of dog and cat muscle to fatigue caused a reduction in both A and B values but that the fall of B was greater than that of A, hence there was a decrease in lactacidogen.

Irving (9) has observed that the effect of stimulation on the lactacidogen content of mammalian muscle was variable but that the increases were more conspicuous.   Prolonged interrupted stimulation usually caused a decrease, however.   Eggleton and Eggleton found ((3) p. 159), "That part of the labile phosphagen-phosphorus which disappears in fatigue is not accounted for by the inorganic phosphate which is liberated."   Our data suggest that this portion not accounted for by the rise in inorganic is that which furnishes the phosphate for Lactacidogen II, because this latter fraction increases on stimulation, while Lactacidogen I, if it undergoes change, tends to decrease.   The following equations illustrate the point.   Phosphocreatine before stimulation, 84; after stimulation, 30; a loss of 54.   Gain in inorganic 40 − 16 = 24.   54 − 24 = 30 to be put into some other fraction. Lactacidogen II before stimulation, 10; after stimulation; 42; a difference of 32.   The calculation for the relationships for the other five experiments between loss in phosphocreatine phosphorus not accounted for as inorganic phosphate and gain in Lactacidogen II is as follows: 30:31, 23:18, 30:29, 12:10, and 14:17.

The order of magnitudes is similar and the differences are within experimental error.

Since Lactacidogen II is relatively acid-stable and hydrolyzes at a rate comparable to the fifth fraction $(T - B)$, yet is hydrolyzed enzymatically, it could be a compound of the hexose ester type.

The experiments show that the total lactacidogen content of rabbit muscle increases on brief stimulation without recovery.

SUMMARY.

1. The hydrolysis of a fresh extract of resting rabbit gastrocnemii by 1 N sulfuric acid in a boiling water bath for 1 hour liberates an amount of phosphate which corresponds approximately to that liberated by enzymatic hydrolysis.

2. In stimulated muscles which were not allowed to recover, the hydrolytic value was lower by 20 to 30 mg. per cent than the enzymatic.

3. Lactacidogen consists of at least two phosphorus-containing compounds. The quantity of one is little affected by brief stimulation, the other is increased.

4. A color test for differentiating ortho- and pyrophosphates is described.

5. When this test was applied to fresh trichloroacetic acid muscle filtrates, they gave negative reactions for pyrophosphate.

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MICROSCOPIC AND X-RAY INVESTIGATIONS ON THE CALCIFICATION OF TISSUE.

By NELSON W. TAYLOR and CHARLES SHEARD.

(From the Section on Physics and Biophysical Research, The Mayo Clinic and The Mayo Foundation, Rochester, Minnesota.)

In recent years a considerable number of papers on the calcification of tissues has appeared in the literature. Attention has been paid to the normal type of calcification as it occurs in the mineralization of growing bones and teeth and also to the numerous instances of pathological calcification, which are of common occurrence. The discoveries in regard to the prominent rôle played by ultra-violet light in development and growth of bone have focused attention on such phenomena as rickets and osteomalacia, in which the deposition of lime salts seems to be insufficient. Exact studies on the metabolism of inorganic substances have been made and the view has gradually developed that there exist certain minimal concentrations of calcium and of phosphate ions in the blood serum which must be maintained for normal health of the animal at its particular state of bodily development.

It has been shown that the concentrations of calcium and phosphate ions may not be varied independently but that they are closely related. Within a considerable range of concentration each ion may be varied without seriously affecting the process of mineralization of the tissues. In order to interpret this on a sound physicochemical basis it has been proposed that the process of deposition of lime salts in the body is simply one of precipitation of a compound of low solubility and that precipitation or solution will occur when the so called ion product of the reacting substances exceeds or is exceeded by the solubility product of the solid phase present. Thus the concentration of calcium ion, for example, may fall to a low value without affecting the deposition of solid calcium phosphate if at the same time the phosphate ion is at high concentration.

When one endeavors to obtain information regarding the solid phase in bone or in teeth or in pathological types of calcium phosphate deposition, the problem presents more difficulties. It is obvious that the nature of the solid phase should be definitely known; otherwise a solubility value for the material has no significance and therefore the critical ion product necessary for deposition cannot be stated precisely. The ionic equilibria may vary in an incomprehensible way if an unnoticed change happens to take place in the nature of the solid phase. The neglect of this point has been the source of much trouble in the literature on solubility and has rendered invalid a good deal of data on solids of indefinite composition. As an illustration one may cite the case of sodium sulfate, which at 20° is soluble in water to the extent of 16.3 per cent Na_2SO_4 by weight if the solid phase is $Na_2SO_4 \cdot 10H_2O$, as compared with 30.6 per cent Na_2SO_4 by weight when the solid phase is $Na_2SO_4 \cdot 7H_2O$.

Analyses of the inorganic part of bones by various investigators have led to interesting results. Holt (1) states that, "The bone matrix consists of a protein substance, ossein or collagen, which constitutes about one-third of its mass. The remaining two-thirds consist of inorganic matter: calcium, phosphorus, carbonate, and magnesium, with perhaps small traces of alkali salts. Only the calcium and phosphorus are present in relatively large amounts, and the ratio in which these elements exist is almost exactly that required to form tertiary calcium phosphate $Ca_3(PO_4)_2$. Thus it is usually stated that about 85 per cent of the inorganic matter of bone consists of this salt, about 14 per cent is calcium carbonate, and the remainder magnesium carbonate and various alkali salts." In support of this statement Howland, Marriott, and Kramer (2) have presented a good deal of experimental evidence to show that the ratio residual Ca to residual P in freshly formed bones as well as in older bones is 1.94 ±0.14, the experimental error being ±3 per cent. This ratio is obtained by assuming that all the carbonate is present in the form of $CaCO_3$ and the small amount of magnesium as $Mg_3(PO_4)_2$. The ratio of residual Ca:P then serves to show the type of calcium phosphate present. For $Ca_3(PO_4)_2$ the ratio is 1.94, while for $CaHPO_4$ the ratio is 1.29. Kramer and Shear (3) report analyses of eleven specimens of normal calcification and seven of pathological calcification. "In all cases the value of the ratio was 1.96 within the experimental error. . . . One type of exception was noted. Three specimens of calcified fibroid of the uterus gave ratios of 2.23, 2.23, and 2.18 respectively.

"The normal adult rats analyzed contained about 15% carbonate calcium, i.e., carbonate Ca/total Ca × 100 was about 15. In the young rats analyzed the carbonate calcium was only from 8 to 10% of the total calcium.

This indicates that the composition of bone in rats is not constant, but changes with age." A later paper by Kramer and Shear (4) lists the analytical results on sixteen specimens of normal rat bone. A mean value of 1.99 ± 0.05 was obtained for the residual Ca:P ratio for these normal bones. In a study on rachitic rats which had been fed cod liver oil concentrate for 8 days and then killed, analyses of the lines of freshly deposited lime salts gave a residual Ca:P ratio of 2.38, while the older shafts gave a ratio of 2.05. The carbonate calcium constituted 16 per cent of the total calcium in the shafts of the test rats. "In these same rats, however, the fresh calcification contained only from 10 to 12% carbonate calcium. Thus the proportion of carbonate calcium in the older bones is 30 to 50% higher than in the fresh deposit. This is similar to the difference between the composition of the bones of older rats as compared with very young rats." Kramer and Shear are of the opinion that the fresh deposit consists of $CaHPO_4$ and owes its excess calcium to calcium hydroxide.

These observations are cited to show that bone is not absolutely constant in its inorganic composition and therefore lacks justification in assigning to it the nature of a single crystalline substance of fixed composition. The close approach of the residual Ca:P ratio to 1.94 has, however, led to the belief that one of the solid phases must be $Ca_3(PO_4)_2$.

Holt, La Mer, and Chown (5) measured the solubility product of tricalcium phosphate in blood serum and in a solution having the same ionic strength as serum, and have compared their result with the corresponding ion product for serum and for cerebrospinal fluid, arriving at the peculiar result that both of these fluids are normally supersaturated with respect to $Ca_3(PO_4)_2$ to a high degree; that is, about 200 per cent in terms of molar solubility. "When fresh sterile blood serum is shaken at 38°C., in the absence of solid $Ca_3(PO_4)_2$ little or no reduction in the ion product occurs, but when the shaking is carried out in contact with this salt a marked reduction occurs in the course of time, quite analogous to that found with inorganic solutions." The authors interpret their result as indicating "the transitory precipitation of secondary calcium phosphate, $CaHPO_4$, and the inherent slowness of the precipitation of $Ca_3(PO_4)_2$, which is perhaps due to the fact that this is a reaction of the fifth order."

Sendroy and Hastings (6) obtained similar experimental results, interpreting them to mean that the excess of calcium corresponding to the degree of supersaturation present is not in ionic form but is bound to some substance or substances which may be of protein character. These authors assume that the solid phases present in bone are $CaCO_3$ and $Ca_3(PO_4)_2$, separate and uncombined in any way. In support of this assumption they made some measurements on the solubility of powdered bone in various salt solutions similar to those used for measurements on $CaCO_3$ and $Ca_3(PO_4)_2$, and concluded that the calcium salts of bone do not behave very differently from these inorganic salts.

Refractive Index Method.

Despite all the work which has been done on the solubilities of $Ca_3(PO_4)_2$ and related problems, there still seems to be uncertainty regarding the nature of this compound. No one has ever prepared pure crystalline $Ca_3(PO_4)_2$ in the laboratory. The product usually obtained is shown by analysis to be richer in lime than corresponds to this formula. As a mineral $Ca_3(PO_4)_2$ is unknown. The only crystalline forms of calcium phosphate described in volume I of the International Critical Tables are cited in Table I.

All these minerals have very low birefringence. Of these brushite is the only one which has been considered a constituent of bone.

TABLE I.
Crystalline Calcium Phosphate.

Mineral.	Formula.	Mean index of refraction (N).
Monetite	$2CaO \cdot P_2O_5 \cdot H_2O$	1.518
Brushite............	$2CaO \cdot P_2O_5 \cdot 5H_2O$	1.545
Martinite	$5CaO \cdot 2P_2O_5 \cdot 1\frac{1}{2}H_2O$	1.606
Isoclasite...........	$4CaO \cdot P_2O_5 \cdot 5H_2O$	1.568
Fluorapatite........	$CaF_2 \cdot 9CaO \cdot 3P_2O_5$	1.633
Chlorapatite..	$CaCl_2 \cdot 9CaO \cdot 3P_2O_5$	1.667
Podolite............	$CaCO_3 \cdot 9CaO \cdot 3P_2O_5$	1.635
Dahllite............	$CaCO_3 \cdot 6CaO \cdot 2P_2O_5$	1.633
Francolite...........	$CaCO_3 \cdot CaF_2 \cdot 9CaO \cdot 3P_2O_5 \cdot H_2O$	1.625

Brushite may be written $CaHPO_4 \cdot 2H_2O$ and thus is seen to be identical with secondary calcium phosphate. It is evident that $Ca_3(PO_4)_2$ (or as it may be written $3CaO \cdot P_2O_5$) is missing from the list. The last five substances named in the list have the CaO and P_2O_5 in the ratio required by $Ca_3(PO_4)_2$. Possibly the crystalline phase present in bone might be one of these compounds, the most probable of which, in the absence of F and Cl, are podolite and dahllite. If such were the case, the percentage of calcium combined with carbonate would be a constant (10 per cent or 14.3 per cent respectively), and the weight ratio of $CaCO_3 : Ca_3(PO_4)_2$ in bone would be 0.108 for podolite and 0.161 for dahllite. It is stated commonly that the ratio in bone is 14:85 or 0.165, although data by Goto (7) on bones of normal rabbits (also quoted by

Sendroy and Hastings) lead to a ratio 0.448. This result would seem to indicate an excess of $CaCO_3$ as such in the bone in addition to the $CaCO_3$ present in podolite or dahllite. It has been emphasized, however, that the inorganic composition of bone is variable and therefore one would hardly expect to find one crystalline substance only. If podolite and dahllite are present in bone, they should be chosen as a basis for solubility and ion equilibrium measurements rather than the so called $Ca_3(PO_4)_2$.

An excellent test regarding the exact nature of the solid phases in the inorganic part of bone is the measurement of the index of refraction of the constituents. The method is rapid and simple in operation and has been used by mineralogists in the identification of the crystalline constituents of rocks. Despite this fact many chemists seem to be either ignorant of the method or unappreciative of its simplicity and general usefulness. The details of the method may be found in *United States Geological Survey Bulletin 679*, entitled "Microscopic Determination of the Non-Opaque Minerals," by E. S. Larsen.

A series of liquids with known indices is prepared. For general mineralogical work these range from 1.45 to 1.74 and differ by 0.01. The indices of refraction of the liquids are determined by an Abbe or Pulfrich refractometer, preferably with a monochromatic light, as, for example, the sodium flame.

Fragments are embedded first in one liquid, then in another, until the index is determined within certain limits. A few fragments are immersed in a drop of liquid on a glass slip, placed on the stage of a microscope, and the iris diaphragm of the substage partially closed so as to reduce the lighting of the field. After focusing sharply on a fragment, the microscope tube is suddenly but slightly raised. A bright line or illuminated zone appears toward the substance with the higher index of refraction. Let us suppose an oil of index 1.60 had been used. If the illumination appears inside the solid particle, the latter has an index greater than 1.60. If it appears in the oil phase, the index of refraction of the particle is less than 1.60. By trying several oils one may find quite readily two adjacent oils in the series whose indices are respectively greater and less than that of the solid particle. By mixing 2 drops of equal size on a glass slip, liquids of intermediate index may be prepared. If the index of refraction of each liquid

has been carefully determined, the index of such a mixture is probably correct to ±0.003. In case the liquid and solid have almost the same value, the border of the particle will appear colored on account of dispersion. In this event it is advisable to use monochromatic light. For this purpose a filter consisting of a saturated solution of potassium dichromate about 1 cm. thick, or a sheet of yellow glass, such as Corning Glass Company G 34-Y, will be found satisfactory.

The optical phenomenon described is known as the Becke effect. Its character may be understood by a consideration of Figs. 1 and 2. Light rays, after traversing a substance immersed in a liquid, will be totally reflected on emergence in case the substance has a greater index than that of the liquid (Fig. 1).
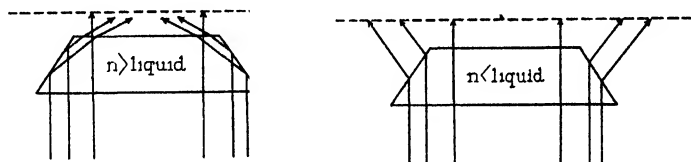
FIG 1.

FIG. 1. Illustrating the Becke effect in measurements on refractive index.
FIG 2. Illustrating the Becke effect in measurements on refractive index.

If, on the other hand, the substance has a smaller index than the liquid, the light rays will enter the liquid and will be refracted as in Fig. 2. If the focus is slightly above the fragment, as shown by the dotted line, there will be a concentration of light toward the fragment in the first case and away from it in the second. Small fragments become completely illuminated or darkened in this way.

A petrographic study of bone has been made by Austin F. Rogers (8). He examined 300 fossil bones ranging in age from the Ordovician up through all the major divisions of geological time to the Recent. The specimens came from twenty-two different states of the United States and from fourteen foreign countries. The following quotations are taken from this paper.

"Nearly all the important zoological groups of vertebrates are represented: ostracoderms, ganoids, selachians, dipnoans, arthrodires, teleosts, a few amphibians, many reptiles (rhynchocephalians, pythonomorphs, sauropterygians, chelonians, crocodilians, dinosaurs, and pterosaurs),

and many mammals (multituberculates, cetaceans, sirenians, artiodactyls, perissodactyls, rodents, carnivores, and primates). . . . . Instead of being silicified, as is the common belief, fossil bones are phosphatized and consist almost exclusively of the mineral *collophane*, a hydrous calcium carbonate-phosphate, which is the chief constituent of phosphorite or so-called phosphate rock. Collophane is an amorphous substance of some-what variable composition, but its properties are sufficiently distinctive to regard it as a definite mineral species, as has been shown by the writer in a recent paper (9)." "The chemical formula of collophane is $3 \ Ca_3(PO_4)_2 \cdot n \cdot Ca(CO_3)(H_2O)_x$ in which calcium is partially replaced by iron, aluminum and magnesium and the carbonate radical by fluorine, the sulfate radical, and oxygen, and when n has the limiting values 1 and 2 and x is also in-definite. The index of refraction of the collophane varies from 1.573 to 1.621, but of those examined about half lie between 1.600 and 1.610. Like other amorphous minerals, the collophane of fossil bone usually shows weak double refraction. The double refraction is doubtless due to strain. It lacks the uniformity characteristic of crystalline substances and the ex-tinction is often wavy. The fossilization of bone consists in the filling of porous bone from which the organic matter of the original bone has been removed during weathering, by calcium phosphate, calcium carbonate and small amounts of other substances During fossilization the specific gravity increases from 1.9–2.0 up to 2.7–2.9 and the index of refraction increases from about 1.56 to 1.58–1 62. The index of refraction of recent bone (weathered bone from which most of the organic matter has dis-appeared) is 1 563 ±0 003. One would expect the collophane of fossil bone to go into solution and to recrystallize in the form of dahllite, $2Ca_3(PO_4)_2 \cdot CaCO_3$, which, next to collophane itself, is the most common mineral found in phosphorites or so-called phosphate rocks, but this rarely happens. The amorphous condition, although theoretically unstable, may persist for an exceedingly long period."

These facts, although dealing with fossil bone, have a bearing on the subject under consideration. If the process of crystallization of collophane into dahllite is so slow that geological time is in-sufficient to change more than a few specimens, we can hardly expect to find large crystals of calcium phosphate predominant in the bony structure of the living animal. It may be noted that the highest indices obtained by Rogers were about 1.62. This is ·of particular interest and will be discussed later.

Experimental Results.

A sample of powder from the cheek bone of a well dried adult human skull gave an index 1.562 ±0.002. A sample from the forehead gave 1.561 ±0.002. Femurs of eight chickens, about 3

months of age, fed on a diet rich in calcium and kept constantly under various selective light filters and irradiated from day to day with ultra-violet light for definite intervals, gave the values shown in Table II. The second set of figures is given in addition to the first set to show the important influence of thorough drying. In every case the index was raised considerably. The figures obtained after 6 days drying are to be taken as correct. The others are without significance except for the purpose of illustration. The details of this research will be published elsewhere by Sheard

TABLE II.
Analyses of Femurs.

Series No.	Treatment.	Index as first determined ($\pm$ 0.003).	After 6 days at 60° ($\pm$ 0.003).
1	Amber glass, 15 min. ultra-violet light, heads only irradiated.	1.541	1.557
2	Amber glass, 15 min. ultra-violet light, bodies only irradiated.	1 552	1.558
3	Amber glass, controls.	1 546	1.549
4	" " 20 min. ultra-violet light, through Corex.*	1.540	1.562
5	Amber glass, 5 min. ultra-violet light, whole bird irradiated.	1.543	1.550
6	Amber glass, 10 min. ultra-violet light, whole bird irradiated.	1.552	1 559
7	Corex control.	1.554	1.559
8	Amber glass, daily in sun 20 min. through Corex.	1.552	1.559

* A special filter made by the Corning Glass Company.

and coworkers. While most of the bones gave indices of 1.559 $\pm$0.003, those from Series 3 and 5 gave 1.550 $\pm$0.003. The difference is about 3 times the experimental error. Chickens of Series 3 received no ultra-violet light but lived continuously under amber glass. Those of Series 5 received only 5 minutes ultra-violet light each day. The chickens of Series 3 and 5 had larger parathyroid glands than any of the others. These comments are introduced to show that the measurement of refractive index may prove to be of considerable value in studies on metabolism. In

any determination of a specimen of bone there are always a few fragments giving indices somewhat smaller or larger than the mean value recorded.

A specimen of a turtle bone had an index 1.550 ±0.003. A sample taken from a well dried femur of a dog gave 1.563 ±0.003.

Dental enamel, as the outermost layer of the teeth, constitutes the grinding and crushing surface and is in direct contact with the fluids of the mouth. It is the first line of defence against decay proceeding from or being initiated in the saliva. Consequently it is worth while to determine the composition and structure of the enamel. Enamel is built up of rods which lie more or less perpendicular to the surface of the teeth. Their thickness is about 4 microns. Between the rods is the so called cement substance. Various writers have speculated on the nature of the rods and cement. The indices of refraction obtained were 1.613, 1.615, 1.618, 1.619, and 1.625 ±0.003 on enamel rods of normal teeth from different sources. These values are a little less than those

Fig 3. Sketches of possible cross-sections of rods.

corresponding to members of the apatite group. Enamel contains small amounts of fluorine (about 1 per cent according to Bertz (10)) but it is probable that the chief mineral constituent approximates dahllite or francolite. However, we again have a certain variability in index, corresponding to the variability in composition observed by several investigators. The difference between enamel rods and bone is striking and will be discussed later.

Considerable attention was paid to the nature of the cement substance. The final conclusion based on the optical phenomena observed is that this is probably organic material calcified to a lesser degree than the rods themselves. Etching experiments were made with 0.2 N HCl and with 1 M lactic acid on fragments containing ten or a dozen rods side by side and a single layer in thickness. It was observed frequently that the end rods would become completely separated from the rest of the group by the action of the acid but would not dissolve for a considerable time thereafter. Cleavage rarely took place across the rods. This indicates that

the space between the rods is structurally more open to attack, as illustrated in Fig. 3.

The presence of organic matter in enamel is demonstrated by its fluorescence in ultra-violet light (11). This fluorescence is . also shown by the enamel in powdered form but is lost when the powder is ashed by heating. Two samples of normal dentine each gave the index of refraction 1.577 ±0.003.

The only conditions of pathological calcification investigated comprised three samples of salivary calculus, commonly known as tooth tartar, and one case of tubercular lung calcification. The three salivary calculi gave indices of 1.564, 1.563, and 1.561 ±0.003. This shows them to be like bone. Analysis of a salivary calculus (from Wharton's duct) by Schuh (12) gave the following results: CaO 39.91 per cent, MgO trace, CO_2 5.34 per cent, P_2O_5 30.45 per cent, H_2O 20.65 per cent. These calculi contained a few calcite ($CaCO_3$) crystals but they were of minor importance. The material as a whole was made up of very small particles and showed no sharp angles or cleavage planes such as characterize ordinary crystals. It has been stated by Naeslund (13) that the microorganisms, *Actinomyces* and *Leptothrix*, are active in forming these concretions in the oral cavity. From the purely chemical side it would be interesting to determine whether the saliva in such mouths is more alkaline than in normal individuals. Since the calculi are like bone, one may wonder whether calculi would not be rare in mouths showing dental periodontoclasia, in which absorption of bone occurs.

A specimen of a tuberculous lung, richly calcified, gave, after thorough drying, the index 1.585 ±0.003. This material was also very finely divided and showed no crystalline character. Until a larger number of cases has been studied it is unwise to make any comment on this result. This work is being continued as specimens of pathological calcification are obtained from time to time.

A single sample of bone from a rachitic rat gave an index 1.561 ±0.003. Six specimens of humeri, from cows kept for a long time on a diet low in phosphorus, which were well dried before testing, gave the following values: 1.562, 1.559, 1.563, 1.568, 1.568, and 1.568 ±0.003. In the main these are slightly higher than the normal of about 1.560, but the difference is not great enough to

indicate a new crystalline species. It is hoped that investigators who have analyzed bones in their possession will measure the indices of refraction in order to establish definitely the effects of each element in the composition. It was pointed out by Rogers that an increase in the content of iron caused an increase in refractive index. Furthermore, additional determinations should be made as to whether or not the value of the refractive index for bone in rachitis is a representative one.

So much attention has been given to $Ca_3(PO_4)_2$ by different writers that we attempted to measure its index. Pure calcium nitrate and potassium phosphate (tribasic) were weighed out in stoichiometric proportions and put into solution. A small amount of KOH solution was mixed with the phosphate to keep it alkaline and the solution of calcium nitrate was then slowly added. The resulting precipitate never settled completely. It was washed several times by decantation and allowed to stand for a month, after which it was filtered, dried, pulverized, and dried again at 80°. When examined microscopically, fairly well defined crystal angles and edges were observed. The index was 1.628 ±0.003. This value would appear to put the material in the apatite group and in view of the method of preparation it may perhaps be represented by the formula $3Ca_3(PO_4)_2 \cdot CaO$. Larsen describes a mineral, voelckerite, having this formula and this index.

Evidence from x-Ray Diffraction Patterns.

De Jong (14) reports the results of x-ray diffraction photographs of modern and of fossil bones. He stated that all the films showed lines characteristic of fluorapatite and of nothing else, but that the various members of the apatite series $3Ca_3(PO_4)_2 \cdot CaX_2$ (where $X_2 = F_2, Cl_2, (OH)_2, O, SO_4$) gave diffraction patterns differing only in details. On the contrary, a specimen of collophane gave a pattern totally different from that of bone. The crystallites in fresh bone are so small that the x-ray fringes are diffuse. "Probably the crystals contain only a few hundred molecules. Most fossil bones give the same lines. On the other hand incinerated bones show sharp lines due to the growth of crystals."

A number of our substances has been subjected to this x-ray test. The materials were finely powdered and placed in thin walled glass tubes which were half filled with pure sodium chloride.

A small wad of cotton separated the two materials.   Each tube
was then exposed to a beam of x-rays for a period of 40 hours and
the diffraction patterns so formed were recorded on photographic
film according to the standard procedure.   Half of the film carried
the NaCl pattern and the other carried that of the substance
under investigation.   The intensities of the spectral lines are
rather weak and the original spectrograms could not be reproduced
satisfactorily.   Hence exact measurements of the positions of
these lines with respect to the NaCl lines have been made, and
Fig. 4 has been constructed from these data.   All the substances
investigated, with the exception of fluorapatite, had been used

Fig 4 Chart of spectral lines prepared from original x-ray spectro-
grams.

for measurements of refractive index.   The fluorapatite, from
Kai, Japan, was a colorless transparent hexagonal crystal.   It
probably contained some chlorine replacing the fluorine, but
otherwise was undoubtedly of high purity.   Its index was found
to be 1.652.

In the spectrograms, similarity of composition is shown by
similarity of pattern in both intensity and positions of the lines.
Small shifts in positions of lines are to be disregarded, since it is
difficult to determine accurately the center of a spectral line which
may be somewhat diffuse, and since, as stated by de Jong, the
various members of the apatite series give diffraction patterns

differing in details.   It is obvious, from an inspection of Fig. 4, that there is very considerable similarity of arrangement of the various patterns.   In general, where relatively strong lines appear in one spectrogram they are to be found in the others, although sometimes of different intensity.   The positions and number of the faint lines are less certain on account of the difficulty of seeing them on the negatives.   It may be concluded that all of the substances, both of normal and pathological calcification, show patterns indicative of an apatite structure, although there are minor variations which possibly may be attributed to the variation of X_2 in the formula $3Ca_3(PO_4)_2 \cdot CaX_2$, or to the presence of magnesium compounds in small amounts or to traces of alkali salts.

DISCUSSION.

There are three lines of evidence which point to the conclusion that the solid phase in bone is composed of minerals of the apatite series $3Ca_3(PO_4)_2 \cdot CaX_2$.   The first is the chemical evidence, which indicates that the residual Ca:P ratio is very close to 1.94. The second is the evidence furnished by the x-ray diffraction patterns.   Finally we have the optical evidence.   In no case did Rogers find indices greater than 1.63.   This is roughly the value for the members of the apatite group.   This upper limit was obtained in specimens in which the organic matter had disappeared almost if not entirely.   The values obtained by us are in the vicinity of 1.56 for bone, 1.58 for dentine, and 1.62 for dental enamel.   This is exactly the order of organic content for these calcifications; that is, greatest for bone and least for enamel. This apparent dependence of the index on the organic content is due quite probably to the fact that each calcified unit or particle in these tissues is encased in a thin organic sheath of low refractive index.   Even if the pure inorganic material has an index of about 1.63 in its central portion, the particle as a whole, when observed microscopically, would appear to have a lower index depending on the water content and relative thickness of this sheath.   The index for water is 1.333.   The presence of this sheath would explain Rogers' observation that the growth of these crystallites into macroscopic crystals is extremely slow.

All of the material examined appeared granular in structure

when viewed under the microscope.   Even in the case of dental enamel, with its very low organic content, each rod was seen to consist definitely of a single row of particles arranged side by side. Photographs of this structure have been published in dental text-books.   Therefore we may represent roughly the calcified particle by two concentric spheres, the inner one containing the apatite mineral of index 1.63 and the outer one consisting largely of organic matter and water and, therefore, of low refractive index. The boundary between these spheres is probably not discontinuous.   The size of the particle as a whole is too small to enable one to distinguish any details of structure.   However, if the cell were poorly mineralized, the inorganic nucleus would be relatively small in comparison to the organic sheath and the bone would show a low index of refraction.   When the cell is extensively calcified, as in the extreme case of dental enamel (97 per cent inorganic), the sheath is very thin in comparison to the interior and the index is high.   In the particular case of calcification of a tubercular lung the index was measured as 1.585, intermediate between 1.55 and 1.63.   It is evident that measurements of the index constitute a rapid method for estimating the degree of calcification of normal or pathological tissues.

If we are right in our contention that the solid phase is an apatite mineral, it is obvious that the solubility relations of this substance will determine the conditions for precipitation in the bone and elsewhere.   The solubility product of such a substance as podolite, $3Ca_3(PO_4)_2 \cdot CaCO_3$, therefore should be used rather than that of brushite ($CaHPO_4 \cdot 2H_2O$) or of the compound $Ca_3(PO_4)_2$.

We are indebted to Drs. L. T. Austin and H. E. Robertson of the Mayo Clinic and to Professors L. S. Palmer and J. W. Gruner of the University of Minnesota for material used in this research.

SUMMARY.

An optical study has been made of several types of calcification of tissues, including normal bone, dental enamel and dentine, rachitic bone, bone low in phosphorus, salivary calculus, and calcification of tubercular lung.   The conclusion is drawn that the solid inorganic phase consists essentially of very small crystals

of *apatite minerals* of the general formula $3Ca_3(PO_4)_2 \cdot CaX_2$, where X_2 ordinarily represents CO_3, F_2, $(OH)_2$, O, SO_4, and Ca, which may to some extent be replaced by Mg. The typical minerals of this formula are podolite, dahllite, and fluorapatite.

We have not obtained evidence to show that the mineral brushite, $CaHPO_4 \cdot 2H_2O$, is present to any extent in either normal or pathological deposits.

The index of refraction may serve as a measure of the degree of calcification of dried tissues, being low for bones and high for dental enamel or in certain cases of pathological deposition. It is probable that the index is determined by the relative amount of inorganic crystal and organic material in the unit structure.

Diffraction patterns of x-rays obtained by the powder method have been obtained for apatite, dental enamel, normal bone, salivary calculus, tuberculous pulmonary calcification, and synthetic tricalcium phosphate. The similarity of patterns indicates similarity of crystal structure. The conclusion is in agreement with that based on the optical evidence.

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THE VITAMIN A, B, AND C CONTENT OF ARTIFICIALLY VERSUS NATURALLY RIPENED TOMATOES.*

By MARGARET C. HOUSE, P. MABEL NELSON, and E. S. HABER.

*(From the Laboratory of the Home Economics Section of the Iowa Agricultural
Experiment Station, with the Cooperation of the
Vegetable Crops Section, Ames.)*

INTRODUCTION.

The use of ethylene in the ripening of fruits and vegetables has received considerable publicity and has been used to a limited extent commercially during the past 3 years. This method possesses distinct advantages over the older method in that it materially reduces the time required for the preparation or finishing for market, is relatively inexpensive, and ripens the product more uniformly. With a consuming public demanding fruits and vegetables at any and all seasons of the year the use of ethylene as a ripening agent has a valuable commercial aspect.

The consumer, however, is interested not alone in the exterior appearance of the final product, but in its nutritive constituents as well. Since fruits and vegetables are eaten largely because of their vitamin value, it is of interest to know the effect this new commercial method of ripening has upon the vitamin content. Accordingly, the vitamin A, B, and C content of tomatoes, a foodstuff commonly subjected to this treatment, was investigated.

HISTORICAL.

The artificial coloring of citrus fruits for the market has been practiced for some time. According to the old method the fruit was placed in rooms or tents heated with kerosene stoves. It was thought that the temperature and humidity thus produced hastened the ripening process, until Sievers and True (1), proved that the combustion products of the kerosene were

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the factors affecting the ripening. In an attempt to determine the particular gaseous constituent responsible for the coloring, Denny (2) found that a number of substances were effective. Many of these were not practical for one reason or another; cost, effect on the fruit, the color produced, the physiological effect on man, *etc.* Ethylene seems to be the best suited to the process and has come into rather general usage.

Ethylene causes an increase in the respiratory rate according to Denny (3), Rosa (4), and Chace and Denny (5), the general consensus of opinion being that ethylene "acts as a stimulus to the normal oxidative processes in the fruit" (4).

According to Rea and Mullinix (6) ethylene can act as a catalyzer converting pure dry starch to reducing sugars in the absence of the enzyme amylase. This conversion can also be brought about by saturating a starch solution with ethylene and allowing the mixture to stand.

Chace and Church (7) found that there was no significant change in the composition of the edible portion of citrus fruits when testing for soluble solids, reducing sugars, sucrose, and citric acid. They noted among other observations that the astringency of persimmons is destroyed by ethylene.

Experiments on the vitamin B content of board-blanched celery and ethylene-blanched celery have been reported by Babb (8). The difference in vitamin content observed was in favor of the ethylene-blanched celery but was not large enough to be considered significant.

Morgan and Smith (9) have tested the vitamin A content of green tomatoes, of tomatoes ripened in diffused light, ripened in ethylene, and ripened in the dark with the exception of three 30 minute periods of ultra-violet irradiation. They report that green tomatoes are relatively poor in vitamin A but that the ripened fruits contain about equal amounts of this vitamin. Their conclusions are based upon observations of two to five animals per lot. It is not possible from the data given to ascertain whether their results are statistically significant or not.

EXPERIMENTAL.

The tomatoes used were of the Bonny Best variety. Twice each week the vines were inspected and three-fourths of the tomatoes that had reached the "green mature" stage (as judged by size, color, and firmness of the fruit) were picked and brought to the laboratory, the other one-fourth being left on the vines to ripen completely. The green tomatoes brought to the laboratory were divided into three lots, the first being fed at the "green mature" stage. The second lot was ripened in air at room temperature (22–25°), and the third lot treated with an ethylene-air mixture. The fourth lot consisted of the tomatoes that were allowed to ripen on the vines.

The third lot (ethylene-treated) was placed on a rack in a tall

bell jar. The jar was provided with inlet and outlet tubes. A constant supply of an ethylene-air mixture, approximately 1:800, was kept passing through the jar at all times. This was done in order that the respiration products might not accumulate and become a factor influencing the speed of the process. Rosa (4) found that a lower concentration, namely 1:4300 (ethylene to air), was somewhat more effective in ripening tomatoes than the higher concentration of 1:800. The higher concentration was used in this study as the object of the experiment was to determine the effect of the ethylene, rather than to decrease the time of ripening to a minimum.

The experimental animals used for the vitamin A and B tests were albino rats 28 days old and weighing between 40 and 50 gm. The technique used was that of Ferry (10), each rat being kept in an individual all metal cage with a false bottom. Daily food consumption records were kept and the animals were weighed once each week or more often. Guinea pigs 6 to 8 weeks old were used for the vitamin C tests. The guinea pigs were weighed twice each week and carefully observed for symptoms of scurvy. Autopsies were performed to determine the severity of the disease.

Vitamin B.[1]—The basal diet used in the vitamin B test consisted of vitamin B-free casein 18, starch 50, butter fat 8, lard 18, salt mixture 4 (11), and cod liver oil 2. The cod liver oil was fed separately from the ration. The rats were divided into four groups of twenty or more animals each, ten males and ten females in each group. No more than four animals of any one litter were assigned to a group. Three animals were used as negative controls and two as positive controls. One group of rats received 4 gm. of green tomato daily for a period of 8 weeks. The second group received 4 gm. of air-ripened tomato, the third 4 gm. of tomato ripened in the ethylene-air mixture, and the fourth 4 gm. of tomato ripened on the vines. By feeding 4 cc. of canned tomato juice Sherman and Grose (12) were able to maintain animals for 8 weeks with an average loss in weight of 2.2 gm. It was estimated that 4 gm. of fresh tomato would support growth near a maintenance level and thus render the method sufficiently sensitive

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[1] Since this work was started the multiplicity of the factor known as vitamin B has been accepted. In this study, the term vitamin B refers to the complex.

to detect small differences in vitamin content. The fresh toma-
toes were eaten readily by the animals. The positive controls
received 200 mg. of dried brewery yeast daily.

The average daily consumption of the basal diet was practically
the same for all groups of animals. The rats fed green tomato
ate 3.2 gm. per day, those fed air-ripened tomato 3.2 gm., those
fed ethylene-ripened tomato 3.2, and those fed vine-ripened
tomato 3.1 gm.
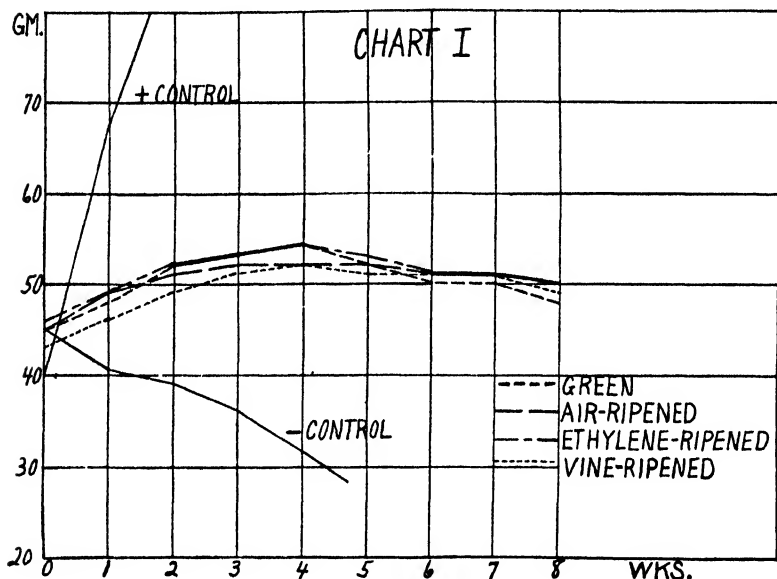
CHART I. Growth curves of rats fed 4 gm. of tomato daily as the sole
source of vitamin B.

The average gain in weight of the group of rats fed green tomato
was 3.2 ± 1.0 gm., of the group fed air-ripened tomato 4.7 ±
0.8 gm., ethylene-ripened tomato 4.7 ± 1.1 gm., and vine-
ripened tomato 6.4 ± 1.2 gm. The growth curves for each
group of rats are given in Chart I. Each curve is a composite
made by averaging the weights of the animals in each group.

The differences in growth in any of the four groups are not
statistically significant. The greatest difference observed was
that between the rats fed green tomato and those fed vine-ripened

tomato. In this case, the mean difference was 1.9 times as large as its probable error and according to Sheppard's probability tables, the chances are only nine to one, that a future trial would give results of the same sign. Since the differences in growth between the animals fed the four groups of tomatoes were not significant, it seems reasonable to conclude that the amount of vitamin B does not differ in green and ripe tomatoes and that the methods of ripening used did not measurably affect the vitamin B content.

Vitamin A.—The basal diet used in this experiment was that recommended by Sherman and Munsell (13) consisting of vitamin A-free casein 20, starch 70, yeast 5, salt mixture 4 (11), and sodium chloride 1. The diet was irradiated for 30 minutes at a distance of 2 feet in order to insure an adequate supply of vitamin D. In this laboratory the stock colony is maintained upon a slightly modified form of the ration recommended by Steenbock (14), 2 per cent of yeast and 10 per cent of wheat germ being substituted for 12 per cent of yellow corn-meal.

Albino rats 28 days old and of known nutritional history were maintained on this diet until their bodily store of vitamin A was depleted. At the end of the depletion period the rats were divided into four groups of twenty animals each. Their average weight at this time was 122 gm. The same precautions as to distribution among the litters and differences in sex were observed as previously. Tomatoes treated in the same manner as in the vitamin B tests were fed to the four groups of animals. Each group received 2 gm. of tomato per animal per day. Both positive and negative controls were used; the positive controls received 10 drops of cod liver oil daily. The average daily consumption of the basal diet by the rats fed green tomato was 10.3 gm., by those fed air-ripened tomato 10.1 gm., ethylene-ripened tomato 10.6 gm., and vine-ripened tomato 10.5 gm. Since the amount of the basal diet consumed was practically the same for all groups of animals it is not possible to ascribe the differences in growth to differences in the food intake.

The group of rats fed green tomato gained 38.5 ± 3.8 gm., those fed air-ripened tomato 57.1 ± 5.7 gm., ethylene-ripened tomato 58.4 ± 5.8 gm., and vine-ripened tomato 55.8 ± 4.6 gm. The growth curves for each group of animals are given in Chart II.

The curves are composites made by averaging the weights of the individual animals in each group.

It is apparent from Chart II that the differences in growth between any of the groups of rats fed ripened tomatoes were negligible. However, the growth of the group of rats fed green tomato was considerably less than that of any of the groups fed the ripened fruit. The mean difference in growth between the group of rats fed green tomato and those fed ethylene-ripened tomato is 3.05
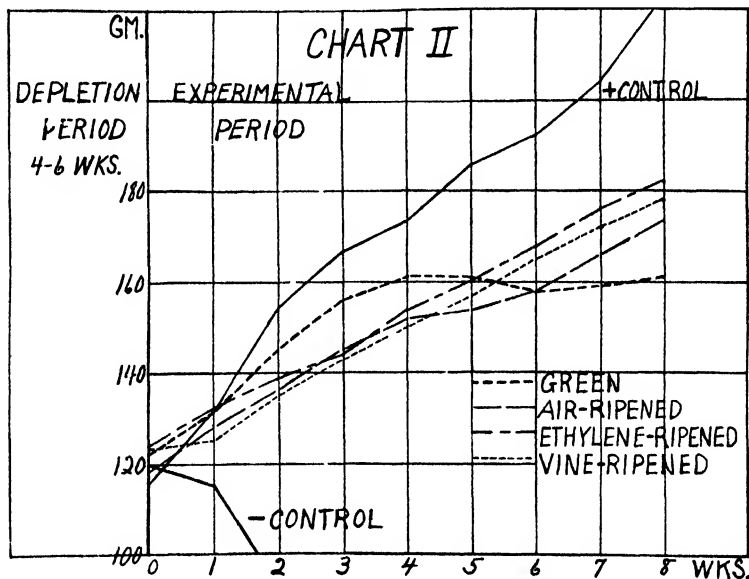
CHART II. Growth curves of rats fed 2 gm. of tomato daily as a sole source of vitamin A.

times its probable error; between the green and vine-ripened tomato 3.00 times its probable error, and between green and air-ripened tomato 3.00 times its probable error. According to Sheppard's probability tables the chances are approximately 49 to 1 that duplicate experiments would give results of the same sign.

Differences from 3 to 4 times their probable errors are regarded as showing a strict significance. Whether or not this is too severe a test for the type of biological data presented above is a matter of controversy (15) From these results it would seem reasonable

to conclude that there is the same amount of vitamin A in the ripened tomatoes, regardless of the method of ripening used, and that the amount found in green tomatoes is somewhat less than that found in the ripened fruit.

Vitamin C.—Guinea pigs 6 to 8 weeks old and weighing between 250 and 350 gm. were placed on a basal diet consisting of a mixture of alfalfa meal and white flour in equal proportions by weight, oats, and water *ad libitum*. During the 1st week after the animals were brought to the laboratory, they were fed, in addition to the basal diet, sufficient amounts of green food to supply an abundance of vitamin C. All animals that did not show a normal growth during this period were discarded. The experiment was begun by withholding the fresh food and feeding weighed quantities of tomatoes as the sole source of vitamin C. From ten to twelve guinea pigs were used in each group Five negative and four positive controls were used, the latter being fed 3 cc. of canned tomato juice daily (16). Group I received 4 gm. of green tomato, Group II 4 gm. of air-ripened tomato, Group III 4 gm. of ethylene-ripened tomato, and Group IV 4 gm. of vine-ripened fruit.

The animals were caged individually, daily food consumption records were kept, and the animals weighed twice each week. Autopsies were performed upon the completion of the experimental period or upon the death of the animal.

The protocols of four typical animals from each group are given. The initial weight, maximum weight, and final weight of the animals, the length of the survival period, food consumption, and autopsy findings are recorded. In Table I, hemorrhage of the joints, of the costochondral junctions, and the looseness of the teeth are indicated by the signs: − (not different from normal), ? (doubtful), and +, ++, and +++ for increasing degrees of severity.

It will be seen from an examination of Table I that the negative controls showed severe scurvy. The positive controls showed no symptoms of scurvy except in the case of the animal which refused to take the tomato juice. The group of guinea pigs fed green tomato showed very definite symptoms of scurvy, more apparent in many cases than in the negative controls. The small quantity of vitamin provided by the green tomato prolonged the life of the animals sufficiently to permit the full development of the characteristic

TABLE I.

Protocols of Guinea Pigs (Male) Fed Tomato as the Sole Source of Vitamin C.

Guinea pig No.	Weight.			Duration of experiment.	Autopsy findings.			Average daily food consumption.		Remarks.
	Initial.	Maximum.	Final.		Ribs.	Teeth.	Hemorrhage of joints.	Mixture.	Oats.	
	gm	*gm.*	*gm.*	*days*						
Fed 4 gm. of green tomato daily.										
962	355	366	212	22	+++	++	+	4.5	10.5	Dull, joints sore.
963	305	310	228	38	++	+++	−	2.8	13.6	Joints sore.
964	347	364	210	37	+++	−	?	3.4	9.6	Diarrhea.
990	306	312	311	77	++	++	−	8.1	8.1	Constipated, joints sore, hind legs wobble.
Fed 4 gm. of air-ripened tomato daily.										
996	268	484	484	77	?	+	−	5.3	11.1	
997	260	260	186	40	+	−	−	2.2	11.1	Constipation early in experiment followed by diarrhea.
1120	300	384	346	65	+	−	−	10.2	10.7	
1121	265	410	410	65	−	+	?	7.5	12.1	
Fed 4 gm. of ethylene-ripened tomato daily.										
981	291	481	466	80	?	+	−	10.4	12.1	
982	297	466	466	80	+	−	−	7.7	14.7	
988	325	516	508	80	+	+	−	10.0	17.1	
1118	260	430	293	62	+	+	?	11.3	9.8	Constipation followed by diarrhea.
Fed 4 gm. of vine-ripened tomato daily.										
971	324	630	620	84	−	−	−	16.3	13.6	
972	300	583	583	84	−	−	−	14.3	13.5	
974	300	550	530	84	−	−	−	12.2	14.9	
977	298	588	560	82	−	−	−	12.6	13.6	
Positive controls; fed 3 cc. canned tomato juice daily.										
957	318	395	380	82	−	−	−	11.2	11.2	
958	330	422	396	82	−	−	−	9.9	14.2	
1115	273	414	411	65	−	?	−	12.0	10.7	

TABLE I—*Concluded.*

Guinea pig. No	Weight.			Duration of experiment	Autopsy findings.			Average daily food consumption		Remarks.
	Initial.	Maximum.	Final.		Ribs.	Teeth.	Hemorrhage of joints.	Mixture.	Oats.	

<table>
<tr><td colspan="11" align="center">Negative controls; fed basal diet only.</td></tr>
</table>

	gm	*gm.*	*gm*	*days*						
952	372	392	216	26	+++	++	+	7.4	9.0	Dull, joints sore, diarrhea.
953	382	390	240	18	+++	+++	++	12 3	5.0	Diarrhea, sore joints.
954	312	347	190	23	++	+++	+	6.1	11.0	Small packed feces, sore joints.
955	327	327	221	17	+++	—	?	8.2	9.3	Constipated.

symptoms. No appreciable difference could be detected between the groups fed the air-ripened or ethylene-ripened tomato. Some of the animals in both groups showed signs of incipient scurvy, others complete protection. They were, however, superior in general health to the group fed green tomato. The guinea pigs fed vine-ripened tomato were by far the healthiest and best conditioned animals of any group. Their bodies were firm and well fleshed, their hair sleek and smooth. Autopsies showed no indications of scurvy.

These data indicate that there is very little vitamin C in green tomatoes, confirming the observations of Hess (17) whose data are unpublished; tomatoes picked green and ripened either in air or in a mixture of ethylene and air contain considerably more vitamin C than the green fruit but not as much as the vine-ripened fruit.

SUMMARY AND CONCLUSIONS.

A comparison was made of the vitamin A, B, and C content of green, air-ripened, ethylene-ripened, and vine-ripened tomatoes. Twenty rats were used in each group for the vitamin A and B tests. Ten guinea pigs were used in each group for the vitamin C tests. Statistical treatment of the data has lead to the following conclusions:

1. The four lots of tomatoes showed no difference in their vi-

tamin B content.   It is evident that the methods of ripening used did not alter the amount of vitamin B present in the green mature fruit.

2. The vitamin A content of ripened tomatoes was found to be greater than that of the green mature fruit.   The same quantity of vitamin A was developed in the tomatoes regardless of the method of ripening used.

3. Green tomatoes were found to be relatively poor in vitamin C.   Air-ripened and ethylene-ripened tomatoes were richer in this vitamin than the green fruit and vine-ripened tomatoes were superior to either the artificially ripened or to the green tomatoes.

4. The commercial method of ripening tomatoes in an ethylene-air mixture produces fruit which is equally as rich in the vitamins A, B, and C as fruit which has been picked green and ripened in air.

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FURTHER OBSERVATIONS ON THE DISTRIBUTION OF ARGINASE IN FISHES.

By ANDREW HUNTER.

(From the Pacific Biological Station, Nanaimo, British Columbia, and the Department of Biochemistry, University of Toronto, Toronto, Canada.)

Some time ago Hunter and Dauphinee (1) reported the results of a quantitative study of the distribution of arginase in some of the tissues, and particularly in the liver and kidney, of fishes. One outcome of this study was to show that, while within any one genus the concentration of arginase in the liver is relatively constant, the differences in this respect between genera, even between genera of the same order, may be remarkably great. Much the highest concentration of arginase was found in the liver of the one elasmobranch examined, the Pacific dogfish. This species occupied in other respects also an exceptional position. Its kidney contained not only, like its liver, more arginase than that of any other fish, but more even than the kidney of a pigeon, and much more than that of a rabbit or cat. Further, while in the ling cod, a teleost, arginase was found *only* in liver and kidney, in the dogfish it existed also in the pancreas, the intestinal and gastric mucosa, the spleen, the spiral valve, the skeletal muscles, and the heart.

The last of these organs—the heart—could be examined in one specimen only, but in this it actually contained about one-fourth as much arginase as the liver, and 4 times as much as the kidney. This rather surprising observation, made almost by accident and only near the end of the investigation, led us to examine, in the same way, the heart of a typical teleost—the herring. In this species also the organ was found to be capable of splitting arginine, and did so at least as actively as the kidney. We had unfortunately no opportunity at that time of confirming these isolated observations or of testing the activity of hearts from other species.

It was left uncertain therefore (1) whether arginase was to be found in the hearts of all fishes, or in those only of certain species or orders, and (2) whether the heart would always, as in our one dogfish, rank next in arginase concentration to the liver. Another question raised, but left unanswered, by our results was whether the exceptional richness in arginase of the dogfish liver was a species peculiarity only, or, as seemed not improbable, represented a general characteristic of the clasmobranchs.

A second residence at the Pacific Biological Station at Nanaimo, B.C., offered an opportunity to take up again these unsettled questions. In doing so I have used the simple and convenient method of arginase determination devised in connection with the earlier work. This consists in (1) the preparation of a standard extract of the tissue under study, (2) the application of this extract to the hydrolysis, under fixed conditions, of an appropriately buffered standard solution of arginine, (3) the decomposition of the urea thus produced by urease, added at an initial pH of 6.8, and (4) the estimation of the urea by colorimetric observation of the change in pH accompanying its conversion into ammonium carbonate. The results are expressed in terms of an arbitrary unit of arginase, defined as that quantity which, acting (at a temperature of 20° and a pH of 7.4) on 6 cc. of a 0.7 per cent solution of arginine hydrochloride containing a $M/20$ concentration of phosphate, will liberate in 24 hours one-tenth of the urea potentially present, or, what is the same thing, will produce a urea concentration of 1 mg. in 5 cc. The details of the whole procedure have been described already (2) and need not therefore be repeated here.

The observations made are collected in Table I, in which, to round out the record, there have been incorporated also a few of Hunter and Dauphinee's earlier data for the dogfish and the herring, together with the previously observed range for the liver in each of the species for which a double set of data is available. The species are named and classified according to the system of Jordan and Evermann (3).

A satisfactory arginase determination requires at least 1.5 gm. of fresh tissue. The heart of a small fish may weigh less than a tenth as much, and even a 20 inch flatfish may possess only 0.6 gm. of cardiac tissue. In testing the arginase content of the

Subclass	Order	Family	Genus and species	Local name	Serial No of fish	Arginase units per oc. in standard extract of: Heart	Kidney	Liver	Previously observed range for liver.*
Selachii.	Cyclospondyli.	Squalidæ.	Squalus sucklii.	Dogfish.	168	34	41	568	213–496
					169	30	21	204	
					198	48			
					55*	109*	28*	496*	
"	Batoidei.	Raiidæ.	Raia binoculata.	Skate.	167	13	40	82	
					164	4	18	436	
Holocephali.	Chimæroidei.	Chimæridæ.	Hydrolagus colliei.	Ratfish.	166		1·6	1·8	
					180	0?	5·6	0?	
					196		4·2	1·8	
Teleostomi.	Isospondyli.	Clupeidæ.	Clupea pallasii.	Herring.	92–93*	8·8*	7·8*	190*	160–190
"	"	Salmonidæ.	Oncorhynchus kisutch.	Coho salmon.	132	8·2	7	57	28–50
					134	4·2	7	142	
					181	2·6			
"	Acanthopteri.	Embiotocidæ.	Phanerodon furcatus.	Silver perch.	171–175	1·1		14	4–11
"	"	"	Tæniotoca lateralis.	Blue perch.	162–163	1·6		2·6	11–12
"	"	Scorpænidæ.	Sebastodes ruberrimus.	Red rock cod.	184–195	·56		45	18
"	"	"	Sebastodes maliger.	Rock cod.	165	0	8·2	78	17–41
"	"	Hexagrammidæ.	Ophiodon elongatus.	Ling cod.	145–146	0		38	12–16
					131	0	9·5	33	
					135–136	0		20	
"	"	Cottidæ.	Scorpænichthys marmoratus.	Sculpin.	137	0		48	11
"	"	"	Leptocottus armatus.	Bullhead.	161	0		28	24–38
					156–160			64	
"	"	Pleuronectidæ.	Platichthys stellatus.	Flatfish.	139–142	0		120	21–25
					170–171			126	
					176–179		6·1		

* Hunter and Dauphinee (1).

heart, therefore, it was often necessary to combine the organs of from two to twelve individuals. The occasions on which this was done, and the number of hearts combined, may be learned from inspection of the serial numbers in the table. It is to be understood that in such cases the liver and kidney were tested in only one individual of the group.

It may be noted in the first place that the concentrations of arginase found in the liver in the present series are often, although not always, higher—sometimes very much higher—than in the earlier one. The explanation of this difference is not clear. A clue may be found in the observation that with some fishes— notably with *Platichthys*—the liver extract very rapidly loses activity, so that if the determination is delayed the result may be far below that given by a perfectly fresh preparation. Some of the earlier results may have been vitiated by failure to observe this phenomenon. On the other hand extracts of salmon liver, to name but one such case, have been found to be relatively stable; and yet even with the salmon the two results now obtained are both above the previously encountered range. It would seem that the arginase content of the liver must be considerably more variable within the species than it at first appeared to be.

In spite of their generally higher level the results with livers still arrange themselves in much the same relative order as before. *Phanerodon* and *Ophiodon* are still among the least active genera; *Oncorhynchus* and *Platichthys* still occupy an intermediate position; and in particular *Squalus*, the dogfish, still appears at the head of the list with a much higher concentration of arginase than any teleost.

The high arginase content of the heart and kidney of the dogfish is also now confirmed. It is true that the earlier figure for the heart appears to have been rather exceptional. Heart and kidney appear now as of nearly equal potency; but both contain, weight for weight, much more arginase than in any of the Teleostomi.

It is possible, further, now to compare with the dogfish another selachian species—the skate. This gave in one example a liver of only moderate activity; but in another—as it happened, a particularly large specimen—the concentration of arginase was of the same unusually high order as in the dogfish. In both cases the kidneys were well above the teleostean range. The hearts

were decidedly less active, but one at least again surpassed the heart of any teleost. It seems fair to conclude that a conspicuous richness in arginase of liver, kidney, and heart is probably a general characteristic of the whole subclass Selachii. Whether other organs showing activity in the dogfish are similarly endowed in the skate was not determined.

It was thought important to compare with the selachians an available representative of the Holocephali, *Hydrolagus* (or *Chimæra*) *colliei*, the ratfish. The Holocephali are sometimes classified along with the selachians as elasmobranchs, and according to Bridge (4) form a "divergent and specialised offshoot from some primitive elasmobranch type, and while retaining most of the essentially distinctive features of their ancestors, have acquired, perhaps independently, certain characters distinctive of Teleostomi, combined with others peculiar to themselves." It will be seen that the position of the ratfish with respect to arginase distribution is apparently as peculiar as its morphology. The organ richest in arginase is usually not the liver but the kidney. In one liver it was impossible to be sure that there was even a trace of the enzyme; in two others there was less than in any other fish liver so far encountered.[1] Whether the heart contains any it is impossible to say. The three specimens were obtained at different times, and none had a heart large enough for a really satisfactory test. The only one with which a test was attempted gave a result no more than doubtfully positive. On the other hand the pancreas, which in the ratfish is a well defined organ of relatively considerable size, gave in one instance at least (Fish 180) unequivocal evidence of the presence of arginase (1.6 units per cc.), and in another (Fish 196) appeared to contain a definite, though hardly measurable, trace. It would seem therefore that in the ratfish arginase, although nowhere highly concentrated, is

[1] It should be said, though, that the examination of the ratfish liver for arginase presented special difficulties. Each of the three examples secured contained a quite extraordinary proportion of oil, which made the preparation of a satisfactory aqueous extract almost impossible. The difficulty was increased by the presence of preformed urea in all ratfish organs, necessitating a special control for each determination. Since, nevertheless, no modification of the method of extraction gave any higher results than those reported, it seems certain that the ratfish liver contains at the most a very moderate concentration of arginase.

rather widely distributed. Possibly this is to be regarded as the primitive condition, out of which, in the course of evolution, there have developed on the one hand the increased concentrations manifested most conspicuously among the Selachii, and on the other the restricted localization usual only among the Teleostomi.

Upon the extent to which, within the latter subclass, restriction has proceeded, the present observations throw some additional light. The herring is found, as might have been expected, to be not the only teleost of which the heart, as well as the liver and kidney, contains arginase. The enzyme is present also in the hearts of *Oncorhynchus*, *Phanerodon*, and *Tæniotoca*. In the hearts of five other genera it could not be detected. We have here therefore a character present in some members only of the subclass. When the position of these members in the usual system of classification is noted, it will be seen that the presence of arginase in the heart is apparently a characteristic rather of the *family* than of the *order*. Families showing it appear in both of the orders represented, but in one at least of these orders there are other families from which it is absent. It may be noted also that the arginase of the heart does not by any means parallel that of the liver. The flatfish has a liver as rich in arginase as that of the salmon, but its heart is inactive; while the silver perch, with a definite supply of arginase in its heart, has one of the feeblest of teleostean livers.

The families of Teleostomi within which an arginase-containing heart has here been observed are the Clupeidæ, the Salmonidæ, and the Embiotocidæ. It has not of course been shown that every species within these three families possesses the characteristic in question, nor yet that in the other families represented in this series it is without exception absent. The most that can be said is that the all too meager data are consistent with such conclusions. It would be interesting to follow the question further, and to include in the survey some of the numerous families and orders of which no representative has yet been examined.

The inquiry naturally suggests itself, whether a teleostean species showing arginase in the heart resembles the selachians further by showing it in other organs also. It has not been possible at this time to attempt to answer this question. One pertinent observation only has been made. The spleen of the

salmon, it has been found, is arginase-free. In the dogfish the spleen contains an appreciable amount of the enzyme. So far as it goes, this observation suggests that, in the Salmonidæ at least, the heart may be the only organ, other than the liver and kidney, in which arginase is present.

SUMMARY.

The exceptional richness in arginase of the liver, kidney, and heart of the dogfish, as previously observed, has been confirmed; and it has been made probable that this is a character common to the whole selachian subclass. The kidney and heart are as a rule much inferior in activity to the liver, and neither one shows with regularity a higher potency than the other.

In contrast with the Selachii, the Holocephali, as represented by the ratfish, have livers very poor in arginase. The highest concentration of enzyme is here found in the kidney. There is some in the pancreas, and possibly a trace in the heart.

Among the Teleostomi an arginase-containing heart appears to be a character confined to certain families. It is found, for instance, in the Clupeidæ, the Salmonidæ, and the Embiotocidæ; whereas in the Scorpænidæ, the Hexagrammidæ, the Cottidæ, and the Pleuronectidæ arginase is restricted apparently to the liver and kidney.

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THE CREATINE CONTENT OF THE MUSCLES AND SOME OTHER TISSUES IN FISHES.

BY ANDREW HUNTER.

(From the Pacific Biological Station, Nanaimo, British Columbia, and the Department of Biochemistry, University of Toronto, Toronto, Canada.)

Available data on the creatine content of fish flesh are few in number and imply surprisingly large differences between different species (1, 2). If early reports are neglected, and those only noted in which the analytical method used was based on Folin's, there are on record just twenty-three determinations of muscle creatine within the class Pisces, as strictly defined.[1] The number of species included in the enumeration is fifteen, but most of these are represented by only a single analysis. The record includes figures as high as 835 mg. per 100 gm.[2] (for *Pagrus major*) and as low as 180 mg. (for the "red" muscles of *Auxis tapeinosoma*) (3). Each of these extremes lies far outside the known range for the muscles of mammals. In view of this situation it seemed worth while to seize an opportunity, recently afforded, of examining, for comparative purposes, the tissues of some of the commoner fishes procurable on the coast of British Columbia. The series secured included fifteen species from thirteen genera, three only of which (*Squalus*, *Oncorhynchus*, and *Raia*) appear in earlier lists. Each genus, with two exceptions, was represented by at least two individuals, some by as many as four. Attention was of course directed chiefly to the creatine content of the skeletal muscles; but in several cases the heart or the testis or both were also analyzed. Another creatine-containing organ, the brain, was examined in two instances only, those of the dogfish and the

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[1] This excludes two analyses of lamprey muscle.

[2] This includes the creatine equivalent of 70 mg. of "free" creatinine, most of which, it may be assumed, existed in the living muscle as creatine.

skate; no other species yielded enough brain tissue for an accurate determination by the method employed.

An entirely just comparison of different species would require that all be obtained and examined under identical conditions. This requirement it was not possible to fulfil. Some specimens were caught by hand-line, others by set-line, others by seining. Others again were taken from a pen, in which they had been confined for varying lengths of time. Those freshly caught without undue struggle were presumably in a normal state of nutrition. Those which had been maintained in captivity probably all suffered from either partial or complete fasting; while those captured in the open were landed in different degrees of muscular exhaustion. These varying conditions were presumably not without influence upon the creatine content of the muscles. In one respect only could all individuals be treated alike. Each, as brought to shore, was killed promptly before asphyxiation by a blow on the head, and the chemical analysis was started in every case immediately after death.

The method of analysis used was essentially the same as that proposed by Baumann and Hines (4) for mammalian muscle, the modifications introduced being similar to those employed by Harding and Eagles (5) in the determination of creatine in brain. From 4 to 5 gm. of tissue, cut up into small pieces, were weighed by difference into a 6×1 inch test-tube, containing 12.5 cc. of 5 N sulfuric acid. The tube was covered by a watch-glass, and heated for 4 hours in a boiling water bath. The product of hydrolysis was filtered, cooled, and made up with the filter washings to a volume of 25 cc. A 10 cc. portion of this diluted extract was then pipetted into a 50 cc. volumetric flask, and its creatinine content determined by following exactly, from this point, the procedure used by Baumann and Hines. The creatinine solution employed as a standard contained 1 mg. of creatinine per cc., and was made by dissolving 1.602 gm. of creatinine zinc chloride[3] in 1 liter of 0 1 N HCl. With skeletal muscle a suitable amount of this standard was found to be 5 cc. (containing 5 mg. of creatinine), and a suit-

[3] The specimen of creatinine zinc chloride employed contained, according to a Kjeldahl determination, 22.90 per cent of nitrogen (theoretical, 23.19 per cent), and yielded, in comparison with a bichromate standard, the theoretical amount of color.

able dilution for the final colorimetric comparison 250 cc. With heart, testis, or brain (employed in the quantity specified) the standard had to be reduced to 1 mg. (1 cc.) and the final dilution to 100 cc. When, as not infrequently happened, the whole amount of such tissue available was less than 3 to 4 gm., these quantities were further reduced to 0.5 mg. and 50 cc. respectively. All results were translated directly into terms of creatine, the assumption being made that none of the tissues contained more than a negligible proportion of free creatinine.

When the procedure described was applied, by way of control, to the mixed flesh from the hind legs of a rabbit, it indicated in two separate 5 gm. samples a creatine content of 0.564 and 0.567 per cent respectively. A 50 gm. sample of the same flesh, treated according to the unmodified method of Baumann and Hines, gave the value 0.564. These results, while above the average for rabbit muscle, are within the range observed by Folin and Buckman (6), and their agreement shows that the small size of the samples used in the present work is not likely to have been a source of error.

This conclusion is strengthened by the outcome of some parallel analyses made on fish material itself. Thus two separate portions of skeletal muscle from Fish 165 (*Sebastodes ruberrimus*) yielded respectively 0.496 and 0.491 per cent of creatine; two others from Fish 156 (*Leptocottus armatus*) gave 0.561 and 0.558; while two portions of cardiac muscle from Fish 150 (*Ophiodon elongatus*) gave 0.167 and 0.163. The excellent agreement of these duplicates showed that reliance might well be placed upon a single analysis, and in the majority of instances a single analysis only was performed.

The species examined and the individual results obtained are listed in Table I, while Table II shows the position of each species in the systematic classification of fishes, together with an average value, calculated from Table I, for the creatine content of its tail muscles. In both tables the nomenclature adopted is that of Jordan and Evermann (7).

In the mammal different muscles are known to present quite frequently considerable differences of creatine content; so that no comparison of species is entirely fair, unless it is based upon analyses of homologous muscles or muscle groups. It seemed

TABLE I.

Creatine Distribution in Individual Fishes.

Genus and species.	Local name.	Serial No. of fish.	Length of fish.	Description of skeletal muscle analyzed.	Creatine per 100 gm. of: Skeletal muscle.	Heart.	Testis.
			in.		mg.	mg.	mg.
Squalus sucklii.	Dogfish.	152	16	Caudal.	625		137
	"	153	15	"	632		169
	"	168	32	"	595		
	"	154	36	"	546	63	
	"	154		Fetal.	460		
Raia binoculata.	Skate.	164	26	Caudal.	486	77	
	"	164		Lateral.	498	97	
		164		Red.	240		
	"	167	36	Lateral.	460		
Hydrolagus colliei.	Ratfish.	166	17	Caudal.	545		152
	"	180	20	"	552		
Clupea pallasii.	Herring.	148	7	"	728	} 214*	
	"	149	7	"	750		
	"	151	8	"	743		
Oncorhynchus kisutch.	Coho salmon.	182	20 5	"	630	} 189	
	"	181	21 5	"	642		
	"	133	21	"		163	
Phanerodon furcatus.	Perch.	147	11	Caudal.	737		117
	"	162	10	"	653		
	"	171	9	"	615		
	"	183	12	"	670		

Tæniotoca lateralis.	Blue perch.	184	11	"	604		
" "	" "	185	10 5	"	622		
Sebastodes maliger.	Rock cod.	145	14	"	531		
" *caurinus.*	" "	146	9	"	527		105
" *ruberrimus.*	Red rock cod.	165	23	"	494		
Hexagrammos superciliosus.	Rock trout.	197	16	"	615		
Ophiodon elongatus.	Ling cod.	138	27	Anterior dorsal.	523	131	133
		138		Midventral.	569		
		138		Caudal.	612		
	" "	143	24	Anterior dorsal.	525	128	144
		143		Midventral.	577		
		143		Caudal.	641		
	" "	131	21	"	575		
	" "	150	46				
Scorpænichthys marmoratus.	Sculpin.	161	23	Caudal.	609	165	138
Leptocottus armatus.	Bullhead.	156	10	"	560		
	"	157	10	"	567		
Platichthys stellatus.	Flatfish.	139	17	"	613		
	"	140	13	"	602		
	"	141	19	"	624		
	"	176	21	"	596		

* For this analysis the ventricles of twelve herrings were combined.

desirable to ascertain at the very beginning how far it might be necessary to apply a similar limitation in the case of fishes.   For this purpose three samples of flesh were taken from each of two ling cod (*Ophiodon elongatus*).   The first was taken from the dorsal region just behind the head, the second from the abdominal wall about midway between the head and the tail, and the third

TABLE II.
Average Creatine Content of Tail Muscles in Different Species.

Subclass.	Order.	Genus and species.	Average creatine content of tail muscles.	No. of specimens averaged.
			per cent	
Selachii.	Cyclospondyli.	*Squalus sucklii.*	0.600	4
"	Batoidei.	*Raia binoculata.*	0.481	3*
Holocephali.	Chimæroidei.	*Hydrolagus colliei.*	0.549	2
Teleostomi.	Isospondyli.	*Clupea pallasii.*	0.740	3
"	"	*Oncorhynchus kisutch.*	0.636	2
"	Acanthopteri.	*Phanerodon furcatus.*	0.669	4
"	"	*Tæniotoca lateralis.*	0.613	2
"	"	*Sebastodes ruberrimus.*	0.494	1
"	"	" *maliger.*	0.531	1
"	"	" *caurinus.*	0.527	1
"	"	*Hexagrammos superciliosus.*	0.615	1
"	"	*Ophiodon elongatus.*	0.609	3
"	"	*Scorpænichthys marmoratus.*	0.609	1
"	"	*Leptocottus armatus.*	0.564	2
"	"	*Platichthys stellatus.*	0.609	4

* Only one of these specimens was from the tail, the other two being taken from the lateral region.

from the compact mass of muscular tissue posterior to the cloacal aperture.   In each instance (see Fish 138 and 143, Table I) there was revealed a progressive and quite conspicuous increase of creatine concentration from before backward, so that much the highest value was found in the powerful propelling muscles of the tail.   Accordingly in practically all other cases the sample for

analysis was taken from the caudal region; and, in computing, for Table II, a series of comparable averages, data from other regions were, with one exception, rejected. The exception was made in the case of the skate, in which the laterally disposed muscles of the pectoral fins, upon which in this form propulsion mainly depends, were found to contain quite as much creatine as those of the tail.

A compilation of all recorded data for the creatine content of normal *mammalian* muscles (1) shows only a few isolated observations which surpass the level of 600 mg. per 100 gm. The mixed flesh of the rabbit, which is relatively rich in creatine, contains on the average only 525. The muscles of most other mammals yield less than 500. A glance at Tables I and II shows that the range of creatine concentrations in the flesh of fishes is in general decidedly higher. In seven out of the thirteen genera represented the average concentration (Table II) is close to 0.6 per cent. In three (*Hydrolagus, Sebastodes,* and *Leptocottus*) it lies between 0.5 and 0.6; in one (*Phanerodon*) between 0.6 and 0.7. In one only (*Raia*) does it fall below 0.5, while there is one also (*Clupea*) in which it exceeds even 0.7.

Earlier estimates have not always indicated for fish muscle such high levels of creatine content as those now reported. Mellanby (8) found only 0.28 per cent in the flesh of the skate, and 0.35 per cent in that of the cod. Similar low values are given by Cabella (9) for *Phycis brasiliensis* and *Atherinichtys platensis*. On the other hand Okuda[4] (3) reports for the shark and the snapper (*Pagrus major*) the quite remarkably high figures of 0.811 and 0.835 respectively. In better accord with the range now observed are the results of Palladin and Wallenburger (10) for *Lota vulgaris* (0.512 to 0.541 per cent), those of Okuda for *Cyprinus carpio* (0.510), *Katsuwonus pelamis* (0.440 to 0.805), *Oncorhynchus tschawytscha* (0.638), and *Thunnus schlegeli* (0.571), and those of Eggleton and Eggleton (2) for *Cottus* (0.410), *Raia clavata* (0.440), and the dogfish (0.460). It is probably a fair comment upon these rather discordant data that the lowest values are the ones most likely to be in error.

The highest figure in Table II (0.74 per cent) is found in one of

[4] The figures quoted as from Okuda include in every case the creatinine equivalent of a considerable quantity of "free" creatinine.

the Teleostomi (*Clupea*), the lowest (0.48) in a selachian (*Raia*). Among the Holocephali, sometimes grouped with the Selachii as elasmobranchs, we find, in the ratfish, the relatively low value of 0.55. On the other hand the dogfish, another elasmobranch, may have as much as 0.63 per cent of muscle creatine, while certain teleostean genera, like *Sebastodes* and *Leptocottus*, may yield no more than the ratfish. There is therefore no sufficient evidence of general systematic differences between any of the major divisions into which the fishes are grouped.

As a matter of fact there may exist within a single order variations nearly as great as any that may be found between members of different subclasses. There is a notable contrast, for instance, between *Clupea* and *Oncorhynchus* among the Isospondyli or between *Phanerodon* and *Sebastodes* among the Acanthopteri. The species itself, indeed, has no sharply fixed content of muscle creatine. Individual perches (*Phanerodon furcatus*), for example, gave figures all the way from 0.615 per cent to 0.737. In spite of this the range for any species is evidently fairly characteristic, and if the example of *Sebastodes* is typical this statement may be extended to include all species within a single genus.

In many fishes, although the bulk of the muscles is almost white, there are found in certain situations sheets or bundles of muscular tissue of a dark red color. The amount of this tissue available is usually rather small; but in the large skate, Fig. 164, it was grouped in conspicuous bundles, easily dissected out, lying ventral to the pectoral fin-rays, and furnishing ample material for a creatine analysis. As indicated in Table I, this red muscle contained just half as much creatine as the pale muscles of the same individual. This confirms similar observations made by Okuda (3) on the red and pale muscles of the bonito (*Katsuwonus pelamis*) and the frigate mackerel (*Auxis tapeinosoma*). It has long been known that the dark meat of rabbits and fowls contains much less creatine than the white.

Another similarity between fish, on the one hand, and mammals or birds on the other, is found in the comparatively low creatine content of developing as compared with adult muscle. This is exemplified by Fish 154, a female dogfish from which were obtained five nearly viable embryos about 7 inches long. The mixed

flesh of these contained only 0.46 per cent of creatine as against 0.55 for the parent fish.

The creatine of living mammalian (or amphibian) muscles, it has been shown, exists for the most part, if not wholly, in a state of combination as "phosphocreatine" or "phosphagen" (11). Upon the removal of the muscle from the body this compound decomposes rapidly into free creatine and phosphoric acid. The phosphoric acid thus liberated constitutes the so called "labile" or "phosphagen phosphorus" of the muscle. In mammalian muscle, therefore, creatine and labile phosphorus occur in equimolecular proportions. If the creatine of fish muscle likewise existed exclusively as phosphocreatine, such muscle should contain not only more creatine than the mammalian, but also more labile phosphorus. Actually it would seem as a rule to contain less. Eggleton and Eggleton (2) report relatively small quantities of phosphagen phosphorus in two out of four fishes examined; while in the muscles of seven different fishes Irving and Wells (12) found no evidence of the presence of any labile phosphorus whatsoever. The precise significance of these findings remains for the present obscure; but evidently it cannot be safely assumed that creatine exists in fish muscle in exactly the same state as in mammalian.

In mammals notable quantities of creatine are known to occur not only in the skeletal muscles but also in the heart, the brain, and the testes. It was to be presumed that in fishes also each of these organs contains creatine, although the only evidence in point is the recent isolation by Steudel and Suzuki (13) of creatinine, as the zinc chloride compound, from the testes of the herring. I have made no attempt at isolation, but the outcome of routine quantitative determinations leaves no reasonable doubt that in fishes as in mammals creatine is a regular constituent of each of the three tissues in question.

In the testes the quantities found (see Table I) are fairly uniform, ranging (in the eight examples secured) between 105 and 169 mg. per 100 gm., with no evident difference between teleost and elasmobranch. The even scantier data available for mammalian testes indicate a probable average in the neighborhood of 200 mg. The fish's testis is apparently less richly supplied with creatine than the mammal's.

For the heart also the nine results obtained with five fish species fall distinctly below the general mammalian level. In thirty-three analyses of cardiac muscle from cats, dogs, rabbits, and sheep Folin and Buckman (6) found creatine percentages between 0.186 and 0.339. In the present series only two figures (for herring and salmon) fall within even the lower part of this range. In the three teleosts represented there seems to be a parallelism between the creatine content of the heart and that of the voluntary muscles. The selachians (or elasmobranchs) occupy, it would appear, a separate position. Both in the dogfish and in the skate the heart has much less creatine than in any of the Teleostomi examined. This, as a matter of fact, is the one point concerning creatine distribution in which there has appeared to be any systematic difference between these two subclasses.

As stated already, the brain was subjected to a creatine analysis in two instances only. The brain of the dogfish, Fish 155, was found to contain 144 mg. of creatine per 100 gm., that of the skate, Fish 164, 116 mg. If these results can be taken as representative, the brains of fishes contain quite as much creatine as those of mammals.

SUMMARY.

The creatine content of the skeletal muscles has been determined in fifteen species of fishes. Considerable differences have been found to exist not only between different species, but even between different individuals of the same species. Each species nevertheless presents a fairly characteristic range of creatine values.

The differences between species do not correspond in any obvious way with zoological subdivisions. In particular there is no systematic difference, with respect to muscle creatine, between the Teleostomi and the elasmobranchs. The latter, however, do show a decidedly lower concentration of creatine in the heart than the former.

In general the skeletal muscles of fishes contain more creatine than those of mammals. Mammals on the other hand show a higher concentration of creatine in the heart and in the testes. The two analyses made of selachian brain tissue indicate a content of creatine equal to that of mammalian brains.

In fishes, as in mammals and birds, red muscles contain less creatine than pale, and fetal muscle less than the adult.

The writer takes this opportunity of thanking Dr. W. A. Clemens, Director of the Pacific Station of the Biological Board of Canada, for placing at his disposal the material and the laboratory facilities which made possible the work described. For the identification of many of the species used he is indebted to Mr. J. R. Dymond.

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THE ASSOCIATION OF VITAMIN A WITH GREENNESS IN PLANT TISSUE.

II. THE VITAMIN A CONTENT OF ASPARAGUS.[*]

BY JOHN W. CRIST AND MARIE DYE.

(From the Department of Horticulture and the Division of Home Economics, Michigan State College, East Lansing.)

Since previous experiments (1) showed that the vitamin A content of head and leaf lettuce varied more or less directly with the greenness of the plant tissue, investigations in which asparagus was used as the source of this vitamin were undertaken. It was thought that certain questions concerning the vitamin properties of asparagus, which arise from the fact that it is offered for consumption in both the green and bleached state and also as a canned product, might be answered.

The experiments have been in progress over a period of 2 years (1927–1928), within which time some of the results from the first season's work have been checked by those of the second. Experimental technique was essentially the same as that employed in the tests with lettuce (1). Albino rats were placed on a vitamin A-free ration, complete in all other dietary essentials, until their store of the vitamin was depleted. Following this, different types and varying amounts of asparagus were fed and growth determined over a period of 8 weeks.

The asparagus was of the Martha Washington variety, from plants growing in the college gardens, these plants being several years old, vigorous, and free from disease. Bleached (term used where tissues have never been allowed to become green) tips were secured, in season, by covering over certain rows with about 4 inches of soil and cutting the stalks just before their emergence above the surface of the ground. When freshly cooked tips were

used, these were prepared each day by cooking with water in a glass beaker just prior to feeding.   The time of cooking was regulated by the softening of the tips, usually being about 15 minutes. Tips for canning were taken from this same bed of plants and canned as follows: blanched 3 minutes in boiling water; packed in tin cans; the cans sealed and then processed for 25 minutes at 10 pounds pressure in a pressure cooker.

Chemical analyses were made by the Experiment Station
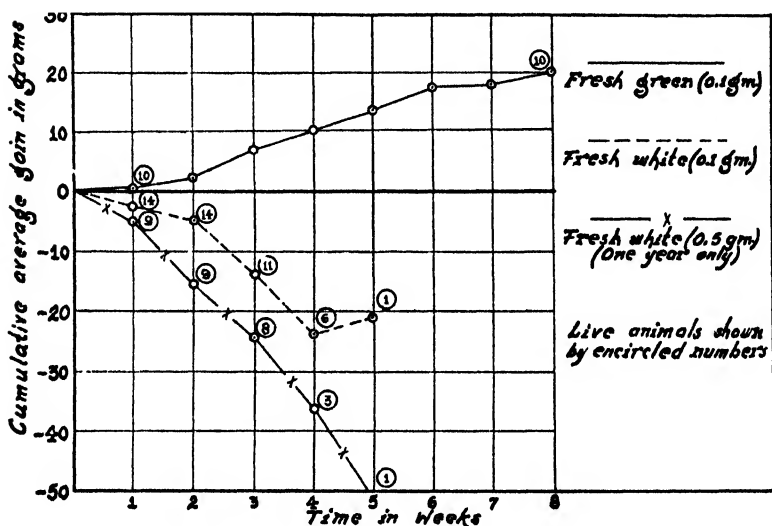
FIG. 1. Growth curves for animals fed fresh green and bleached asparagus.

Chemist after the following methods: nitrogen, Kjeldahl-Gunning-Arnold; sulfur, Association of Official Agricultural Chemists magnesium nitrate; manganese, Willard and Greathouse; iron, Association of Official Agricultural Chemists colorimetric; calcium, Shohl modification of McCrudden; phosphorus, Briggs' modification of Bell-Doisy.

Fresh Asparagus, Green and Bleached.

In both years, the daily amount of fresh green asparagus fed to each animal was 0.1 gm.   In the 1928 experiments there were

two lots of animals on fresh bleached asparagus, one of which had a daily quantity of 0.1 gm. and the other 0.5 gm. The part of the stalk from which the portion for feeding was taken was the same in all the experiments, the second internode back of the tip end. Fig. 1 shows the combined results for the 2 years. Negative controls consisting of one animal from each litter used were run in every case, but are not included on the graphs since without exception the animals continued to lose weight and death ensued.

TABLE I.

Water in Fresh Tissue, and Partial Chemical Analyses (Oven-Dry Basis) of Asparagus Tips.

Sample.	Water content	Ash.	N	Fe	Mn	S	Ca	P
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Bleached (entire stalk).	93 2	7 72	4 62	0 0120	?	0 441	0 175	0 632
Green " "	91 7	8 69	6 36	0 0130	0 001	0 600	0 254	0 822
Bleached (entire stalk).	92 2	6 42	4 15	0 0231	?	0 351	0 176	0 570
Green " "	91 1	9 51	5 87	0 0119	0 001	0 512	0 244	0 761
Green*(bleached base)...	93 2	6 98	3 45	0 0199	Trace.	0 344	0 153	0 509
" (green tips). .	91 8	8 63	5 76	0 0147	"	0 563	0 205	0 755
Green* (bleached base).	92 2	6 74	3 58	0 0164	Trace	0 317	0 132	0 504
" (green tips).	91 5	8 68	5 96	0 0153	"	0 478	0 212	0 780

* Parts of the same stalk.

Fig. 1 shows clearly the superiority of the fresh green asparagus. The cumulative average gain was 20 gm., with no deaths. As regards the animals fed bleached asparagus (fresh), decline in health and weight was continuous and all were dead at the end of the 5th week. This held true for those fed 0.5 gm. daily (1 year's work, 1928) as well as for the lots fed 0.1 gm.

Partial chemical analyses of the fresh asparagus used in each of the two experiments were made. The order of the results for the 2 years was the same, and hence only those for 1927 are presented. These are given in Table I.

Table I shows that the bleached asparagus, whether as such or

as the bleached basal portions of green stalks, was always higher in water content and iron but lower in ash and also in each of the several other elements determined, excepting possibly manganese. The quantities of manganese were so small that, though the very slight differences favored the green tissue, they cannot be taken as significant.

Cooked Asparagus, Green and Bleached.

The daily ration of cooked asparagus for the animal was 0.1 gm. Fig. 2 graphically summarizes the combined results of the 2 years work.
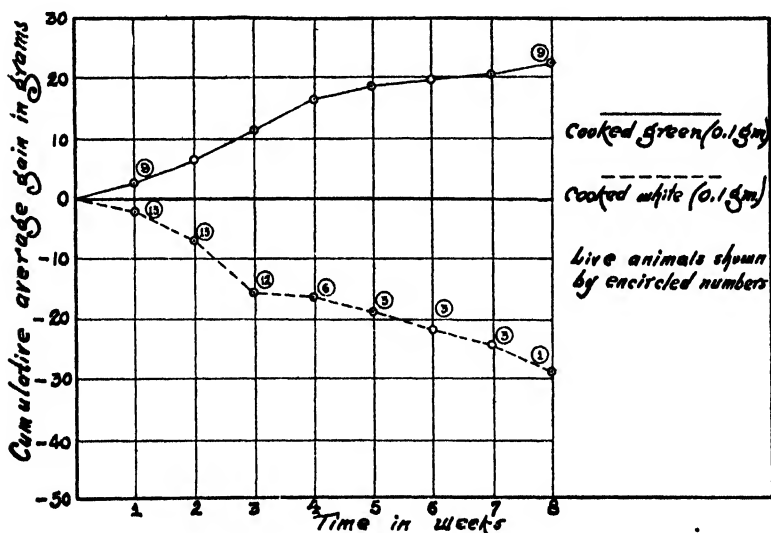
FIG. 2. Growth curves for animals fed cooked green and bleached asparagus.

Fig. 2 demonstrates the higher vitamin A content of the cooked green asparagus as compared with the cooked bleached. The relationship is not identical with that which obtained when the two kinds of asparagus were fed in the fresh state (Fig. 1).   Animals fed on the bleached cooked product fared better than those on the same amount of fresh bleached.   This difference has occurred consistently in all of our experiments.

Canned Asparagus, Green and Bleached.

This was a single experiment, performed in 1927 and not repeated the following year. Animals on canned green asparagus were fed 0.1 gm. daily, those on canned bleached asparagus 0.2 gm. daily. Fig. 3 shows the results.

The animals on canned green asparagus promptly recovered and made good growth, while those on the canned bleached
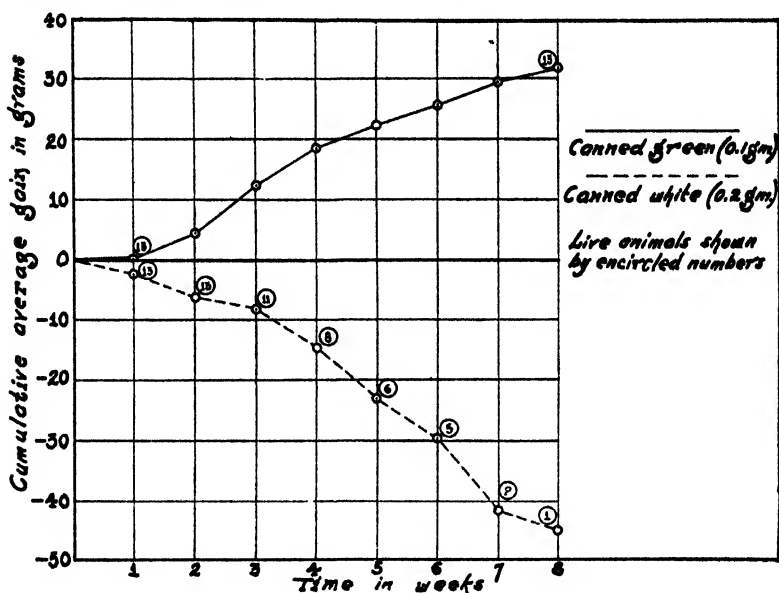
FIG. 3. Growth curves for animals fed canned green and bleached asparagus.

product kept declining and died at a rapid rate, after the 2nd week. This happened even though they received daily a quantity of the material double that provided for the other group.

DISCUSSION.

Again, as was shown formerly in the experiments with head and leaf lettuce (1), it appears that there exists a positive correlation between the degree of greenness in edible plant parts and their content of vitamin A. Differences between green and bleached

asparagus were even more pronounced than obtained with the two types of lettuce.   There is no direct evidence that the chlorophyll is the vitamin, nor that it would be impossible to create circumstances wherein the vitamin might be synthesized and made present in abundance though chlorophyll were .not developed in appreciable quantity.   However, the fact remains that where the tissues are decidedly green the vitamin is abundant. Whether or not the chlorophyll or some part of the chlorophyll molecule, as say the phytol alcohol unit, is the vitamin, or functions in production of the vitamin, or is merely a circumstance attendant upon the reactions of the plant when environed so as to effect the synthesis of the vitamin is an open question.

Moore's experiment (2), together with those of some other investigators whose publications are reviewed in his article, seemed to show that light is not necessary to the formation of vitamin A.   Consequently, chlorophyll which forms in the light, unless the plants are denied some other factor essential to its formation (3), is not required.   His contention is that etiolated wheat shoots contain vitamin A but in a quantity smaller than that of normally green shoots.   Such shoots restore growth when fed in larger amounts.   He fed rats at the rate of thirty shoots per day (equivalent to 1.5 gm. of dry wheat).   Nevertheless, in our experiments, increases in the quantity of bleached asparagus fed to rats, from 0.1 to 0.2 gm. (canned) and even to 0.5 gm. (fresh) did not serve to bring about recovery, promote growth, and prevent death.

In considering this problem it is well to bear in mind (a) that chlorophyll may exist in elemental, more or less colorless form (4), and (b) that either the etiolation or bleaching of plants and plant parts leads to pecularities in their chemical composition. Perhaps, the most obvious and important aspect of this altered chemical composition is the presence of amino and amido bodies such as glutamine, tyrosine, arginine, leucine, histidine, lupanine, trigonelline, and even choline and betaine either *de novo* or in quantitative proportions much greater than what is normal where neither etiolation nor bleaching has been accomplished.   Numerous investigations have shown this to be true.   Though light may not be absolutely necessary to protein synthesis when the plant

is adequately supplied with carbohydrates and available inorganic nitrogen (5), it remains true that in naturally etiolated and bleached plant parts there is an extra large accumulation of amino and amido compounds which are not promptly synthesized into the respective proteins. Experiments (6) have shown that when at least some of these intermediate compounds are ingested, especially in unduly high concentrations, by animals, their metabolism is unfavorably affected. If these considerations are taken into account, it is relevant to infer that the poor quality of bleached asparagus as food for the animal may not be due alone to vitamin A deficiency but also to an overabundance of deleterious chemical compounds. This conclusion has some additional support in the fact that cooking the white asparagus improved its nutritive value to some extent; a result which could have been due to the effect of the cooking process on the chemical constituents of the tissues.

That a positive relationship exists between chlorophyll development and vitamin A content seems to be established. Whether or not this association is unalterably one of cause and effect is a question still to be answered, and probably hinges upon the experimental task of procuring plant tissue which is non-green, and yet whose chemical composition is not such as to introduce factors which complicate the results.

SUMMARY.

1. Green asparagus, whether fresh, freshly cooked, or canned, when fed daily at the rate of 0.1 gm. per animal, contained vitamin A in quantity sufficient to promote health and growth in albino rats.

2. Fresh bleached asparagus, when fed daily at the rate of either 0.1 or 0.5 gm. per animal gave no stimulus to health and growth. The animals died as rapidly as the negative controls.

3. Cooking in open kettle fashion effected an improvement in the nutritive quality of bleached asparagus, though not rendering its value comparable to that of the green product cooked in the same manner.

4. Green asparagus tissue had lower percentages of water and iron than the bleached tissue, but higher percentages of ash, nitrogen, sulfur, calcium, phosphorus, and possibly manganese.

5. The data from these experiments support the conclusion that the vitamin A content of plant tissue is associated with its greenness.

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PROTEINS OF THE AVOCADO (PERSEA AMERICANA MILL).

By D. BREESE JONES AND CHARLES E. F. GERSDORFF.

(From the Protein and Nutrition Division, Bureau of Chemistry and Soils, United States Department of Agriculture, Washington)

In this paper are described the isolation and composition of some proteins of the avocado. Data are given concerning the proteins of a type of vegetable tissue that has been very little investigated. Aside from the work of Smith (1) on the orange there has been to our knowledge nothing published regarding the nature and properties of the proteins of fruits.

In addition to the scientific aspects connected with a study of the proteins of the avocado, the growth of the avocado industry in the United States during recent years, and the increasing popularity of this fruit as an article of food lend further interest and importance to a consideration of the proteins from the standpoint of their biological value.

Proteins of three different types have been isolated from the fresh avocado. One was removed by extracting the fresh fruit with 10 per cent sodium chloride solution. This protein was separated from the saline extract by three different methods; heat coagulation (Preparation I), precipitation with ammonium sulfate (Preparation II), and acidification with acetic acid (Preparation III). The close agreement between these preparations obtained by different methods, with respect to their properties and composition (Table I), strongly indicates that they represent one and the same protein. This protein behaves in most respects like a globulin. It is soluble in salt solution, from which it can be precipitated by addition of ammonium sulfate to 67 per cent of saturation, or by addition of acetic acid to pH 3.9. On the other hand, it cannot be precipitated from a 5 per cent sodium

chloride solution by dilution with 25 volumes of water, nor have we been able to separate it by dialysis.

Another protein fraction was obtained which was insoluble in sodium chloride solution but soluble in an alcoholic solution of sodium hydroxide. By slightly acidifying the alkaline solution with acetic acid the protein separated as a flocculent precipitate (Preparations IV and V).

The slightly acid alcoholic filtrates from Preparations IV and V yielded on dilution with water another protein fraction (Preparations VI and VII).

TABLE I.

Elementary Composition of Proteins. *

Preparation No.	N	C	H	S	Moisture.	Ash.
	per cent	per cent	per cent	per cent	per cent	per cent
I	15.27	53.12	8.56	2.82	6.52	0.42
II	15.37	52.61	8.05	2.87	3.92	1.03
III	15.30	53.03	8.10	2.97	3.60	0.59
IV	13.41	56.49	7.06	2.02	4.04	1.12
V	13 44	56 58	7 13	2 06	5 01	4 91
VI	16.32	55.52	7.40	1.90	3.92	2.86
VII	16.14	55.34	7.43	1.88	6.40	1.49

* The percentages are calculated on an ash- and moisture-free basis, and with the exception of those for sulfur, moisture, and ash, represent average results of duplicate analyses.

The method of preparing the fraction obtained by acidifying the alkaline alcoholic extract (Preparations IV and V) suggests a type of protein having somewhat the properties of glutelins. However, in view of the enzymic activity present in the pulped fruit of the avocado, it is possible that some of the salt-soluble protein originally present had become denatured, and thus escaped extraction with the salt solution, to be later dissolved from the residue by the alcoholic alkali extractant. It is also possible that this fraction represents a type of protein hitherto uncharacterized, and which does not fit into the generally accepted classification of proteins.

It is of interest to note that Preparations VI and VII were separated from an alcoholic solution, although practically no protein could be extracted with alcohol from the fresh avocado.

These preparations may represent a secondary product formed by the action of the alkali upon an original protein of more complex structure.

TABLE II.

*Distribution of Nitrogen in Avocado Proteins as Determined by the Van Slyke Method.**

Nitrogen.	Preparation No.			Amino acids expressed in percentages of proteins.†			
	I	V	VI	Amino acid.	I	V	VI
	per cent	per cent	per cent				
Total N...............	15.27	13.44	16.32	Arginine........	7.94	4.46	12.94
Amide ".............	6.03	8.91	5.35	Histidine........	0.59	2.04	0.99
Humin N absorbed by lime...........	3.15	7.49	5.06	Lysine...........	7.06	6.71	3.95
				Cystine.	2.03	1.84	1.80
Humin N in amyl alcohol-ether extract...............	0.16	0.11	0.44	" ‡.........	1.48	0.11	
				Tryptophane§....	2.12	0.38	1.07
Arginine N.........	16.74	10.67	25.51	Tyrosine‖........	7.01	4.92	2.81
Cystine "........	1.55	1.60	1.28				
Histidine "........	1.04	4.10	1.64				
Lysine "	8.86	9.57	4.64				
Amino N of filtrate..	60.47	55.38	48.12				
Non-amino N of filtrate.	2.88	3.04	6.90				

* Nitrogen figures are corrected for the solubilities of the phosphotungstates of the basic amino acids.

† Except as indicated, the percentages are calculated from the average results of duplicate analyses by the Van Slyke method.

‡ Determined by Dr. M. X. Sullivan according to the Sullivan colorimetric method.

§ Determined by the method of May and Rose.

‖ Determined by the colorimetric method of Folin and Looney.

The three different proteins isolated are clearly differentiated from one another both by their elementary composition (Table I) and by the distribution of nitrogen (Table II).

The avocados[1] used in the work described in this article were grown in California and were mostly of the Fuerte variety. The

[1] The avocados were furnished by the Calavo Growers of California, and were of the grade that are marketed under the trade name "Calavo."

edible portion contained about 71 per cent water, 20 per cent oil, and 0.38 per cent nitrogen, equivalent to 2.37 per cent crude protein (N × 6.25). The proteins actually isolated amounted to 0.67 per cent of the fruit, and represented 28.5 per cent of the total nitrogen. In its content of nitrogen the avocado leads almost all fresh fruits.

A meal prepared by drying the fresh fruit at room temperature, and extracting the oil with ether was found to be unsuitable for a study of the proteins on account of changes that had occurred in the proteins, caused apparently by the action of enzymes. It was therefore decided to abandon the idea of preparing a meal, and to work directly with the fresh fruit.

It was found that three successive extractions of the fresh pulp with 10 per cent sodium chloride solution removed 39 per cent of the total nitrogen, and that 32 per cent of this nitrogen was contained in the coagulum formed by heating the salt extract.

The residue that had been extracted with sodium chloride solution still contained 39 per cent of the original nitrogen of the meal that was extractable by boiling 0.1 N, 60 per cent alcoholic solution of sodium hydroxide. By neutralizing the alkaline extract with acetic acid a precipitate formed which contained 26 per cent of the total nitrogen of the meal.

By successive extractions of the fresh avocado with 10 per cent sodium chloride solution and alcoholic sodium hydroxide, 78 per cent of the total nitrogen was removed, 58 per cent of which was protein nitrogen precipitable by tannic acid.

Preparation of Proteins.

The edible portion of the fruit was finely comminuted and extracted with 10 per cent sodium chloride solution. The clearly filtered, light brown salt extracts were neutral to litmus, but on standing they gradually turned darker and became acid in reaction, with the separation of a small quantity of protein, which completely dissolved when the solution was neutralized with 0.1 N sodium hydroxide. When slowly heated the sodium chloride extracts yielded a flocculent precipitate at 68°. Continued heating of the extract up to boiling gave no further significant amount of coagulum.

Heat-Coagulable Fraction. Preparation I.—The salt extracts

were heated to boiling, and the coagula were thoroughly washed, first with distilled water which had been slightly acidified with acetic acid, then with boiling distilled water, and finally with boiling 95 per cent alcohol. These washings were repeated many times. The protein was then dried in the usual way with absolute alcohol and ether. The final product consisted of a light, cream-colored powder which weighed 22 gm. This quantity represented the yield obtained from 5 kilos of avocados.

Fraction Precipitated by Ammonium Sulfate. Preparation II.—To the clear, filtered, sodium chloride extract of 3422 gm. of avocados was added enough ammonium sulfate to make the extract 67 per cent saturated with this salt. The precipitate which separated was dissolved by addition of water. It had been previously found that this protein could not be separated by dialysis. An attempt to separate the protein by heat coagulation gave a finely divided precipitate which could not be removed by filtration or centrifugalization. Addition of 5 volumes of 95 per cent alcohol, however, caused the separation of a flocculent precipitate. The thoroughly washed and dried product weighed 4 gm.

Fraction Precipitated by Acidification of Sodium Chloride Extract with Acetic Acid. Preparation III.—Acidification of a 10 per cent sodium chloride extract of the avocado with acetic acid gave the maximum precipitation at pH 3.9. The washed and dried precipitate obtained in this way from 1618 gm. of avocado weighed 4 gm.

Alcoholic Alkali-Soluble Fraction. Preparations IV, V, VI, and VII.—The residue remaining after exhaustive extraction of 7 kilos of avocado pulp with 10 per cent sodium chloride solution was boiled for 10 minutes with a 0.1 N, 60 per cent alcoholic solution of sodium hydroxide. The alkaline extract was readily filtered through a folded filter paper, and the clear filtrate was acidified with acetic acid. The flocculent precipitate which separated was washed many times with distilled water which had been slightly acidified with acetic acid, and was finally washed with hot 50 per cent alcohol. After drying in the usual way, 7 gm. of light material were obtained (Preparation IV).

In a similar way, Preparation V (12 gm.) was obtained from 5040 gm. of avocado.

The slightly acidified, 60 per cent alcoholic filtrate from Prepara-

tions IV and V still contained considerable protein which was precipitated by addition of several volumes of water. The dried and purified precipitates (Preparations VI and VII, respectively) consisted of light colored powders. Preparation VI weighed 4 gm.

The elementary composition of the different protein fractions obtained as described is given in Table I.

Preparations I and IV were obtained from a lot of avocados of the Taft variety. The other preparations were made from the Fuerte variety.

Analyses of Proteins by Van Slyke Method.—The general properties and elementary composition of the six preparations described indicate that we were dealing with three different proteins. No essential difference was observed between Preparations I, II, and III, although prepared by different methods. Preparations IV and V were practically identical in their behavior and composition, but distinctly different from the preceding. Preparations VI and VII were alike, but differed from all the others. As representatives of the three proteins, Preparations I, V, and VI were analyzed by the Van Slyke method. The results are given in Table II.

Determinations of Tyrosine, Tryptophane, and Cystine.

The determination of tryptophane was carried out essentially by the colorimetric method of May and Rose (2). Tyrosine was determined colorimetrically by the method of Folin and Looney (3). In addition to the values found for cystine by the Van Slyke method, there are also included in Table II figures for this amino acid obtained colorimetrically. In connection with a comparative study of methods for the estimation of cystine, Dr. M. X. Sullivan of the Hygienic Laboratory, estimated the cystine in Preparations I and V according to the colorimetric method developed by him (4) which has been shown to be highly specific. In view of the well known uncertainty regarding values for cystine as determined by the Van Slyke method, and the high degree of specificity shown for the Sullivan method, the percentages obtained by the latter method are to be regarded as expressing more accurately the actual amount of cystine present in the proteins. This view is strongly supported by correlations observed in connection with the results

of feeding experiments carried on in this laboratory with certain cystine-deficient proteins, and the cystine content of these proteins as determined by these two methods.

SUMMARY.

Three different types of protein have been isolated from ripe avocados, *Persea americana* Mill. By extraction of the edible portion of the fruit with 10 per cent sodium chloride solution a protein was obtained which behaved in most respects like a globulin. In the saline solution it coagulated at 68°, and was precipitated both by addition of acetic acid and by making the solution 67 per cent saturated with ammonium sulfate   It contained 15.31 per cent nitrogen.

By slightly acidifying with acetic acid an alcoholic alkali extract of the residue remaining after the extraction with sodium chloride, a second protein containing 13.42 per cent nitrogen was obtained. Dilution of the slightly acid, alcoholic filtrate with water caused the separation of a third protein fraction. This protein contained 16.23 per cent nitrogen. Elementary composition and distribution of the nitrogen by the Van Slyke method in the proteins were determined. Cystine, tryptophane, and tyrosine were determined colorimetrically.

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FACTORS IN THE METABOLISM OF LACTOSE.

IV. THE DISPOSAL OF LACTOSE ADMINISTERED TO THE RABBIT.*

By RALPH C. CORLEY.

(From the Department of Bio-Chemistry of the School of Medicine, Tulane University, New Orleans.)

For several years there has been employed a method for the determination of certain sugars, dependent upon their resistance to fermentation by yeast, the method probably being applicable to any unfermentable sugar. It is desired to report observations on the disposal of lactose administered to the rabbit. The methods and the experimental procedure employed have been those previously described (Corley, 1928). Lactose was found to have about the same resistance to fermentation as galactose. 96 to 103 per cent of lactose added to blood could be recovered. Results are reported in terms of glucose, lactose having 55 per cent of the reducing power of this sugar.

In one study of the effect of tartrate poisoning urinary volume was not noticeably decreased and sugar excretion occurred. The severity of the intoxication may be indicated by the early death of the rabbit. This protocol (Rabbit 27, Table I) has been included in the tabulations because it is felt to be of interest when compared to the usual findings. With the exception of this and of those in which the intestinal contents were analyzed, each of the experiments recorded is typical of several quite concordant results.

Following the intravenous injection of 1 gm. of lactose, the unfermentable reducing substances of the blood returned to normal in 3 hours. Within this period over 75 per cent of the administered lactose (767 mg., 422 mg. as glucose) was accounted

for by the urinary reducing substances resistant to fermentation. The simultaneous administration of insulin was not found to have any striking effect on the rate of removal of circulating lactose. The experiment presented gave at 1 and 2 hours the lowest values for the unfermentable reducing substances that have been obtained in several similar experiments. In tartrate nephritis, when there was practically complete suppression of the urine, after an initial drop, for several hours there was very little change in the

TABLE I.

Effect of Intravenously Injected Lactose.

Rabbit No................ Weight, *kg*............... Lactose administered, *gm* Time of fasting, *hrs*......	6 1.80 1.0 42		27 1.80 1.0‡ 48		111* 2.00 1.0 48		27† 1.80 1.0 96	
Blood sugar, *mg. per 100 cc.*	Total.	Unfer- ment- able.	Total.	Unfer- ment- able.	Total.	Unfer- ment- able.	Total.	Unfer- ment- able.
Control period.	103	31	107	30	110	31	94	27
After lactose ad- ministration.								
3 min.	261	170	226	157	234	162	254	170
1 hr.	139	60	66	47	197	107	180	79
2 hrs.	139	39	74	30	197	101	178	58
3 "	120	30	104	27	182	101	154	33
Urine sugar, *mg.*		422		380		0		351

   * 2 gm. of tartaric acid in molecular solution of Na_2CO_3 subcutaneously 16 hours before.

   † 1.80 gm. of tartaric acid in molecular solution of Na_2CO_3 subcutaneously 14 hours before. Dead 15 hours later.

   ‡ Plus 2 units of insulin.

residual reduction of the blood. However, with little if any effect on the rate of elimination by the kidneys (Rabbit 27, Table I) the rapidity of removal of the lactose from the blood was rather comparable to that observed in the normal rabbit. The ingestion of lactose had but little effect on the residual reduction of the blood. Smaller doses of lactose had little influence on the total sugar of the blood, but larger amounts at times caused an evident hyperglycemia. Analyses of the intestinal contents may be taken to indicate that absorption had occurred.

The interpretation of results obtained with lactose is complicated by its nature since as a disaccharide it is capable of splitting into the constituent monosaccharides. Folin and Berglund (1922) found that after lactose ingestion, lactose as well as galactose appeared in the urine. It is rather generally accepted that there is an absence of a circulating lactase in the blood, the great difference between the ease of utilization of intravenously injected

TABLE II.

Effect of Enterally Administered Lactose on Blood Sugar.

Rabbit No.	26		29		5		43		110	
Weight, *kg*	2 50		2.30		2.10		3 00		2.00	
Lactose administered, *gm.* ..	2 40		5.00		6.30		9 00		6 00	
Time of fasting, *hrs* ..	72		72		72		96		134	
Blood sugar, *mg. per 100 cc.*	Total.	Unfermentable.	Total.	Unfermentable.	Total.	Unfermentable.	Total.	Unfermentable.	Total.	Unfermentable.
Control period.	79	25	81	36	95	36	94	33	126	25
After lactose administration.										
1 hr.	78	27	93	43	141	27	110	36	139	25
2 hrs.	88	24	99	36	150	31	101	30*	123	25†
3 "	103	43	110	36	128	30				
4 "	103	22	115	30	140	22				
Urine sugar, *mg.*	48	48	40	40	44	44				

* Animal killed. Intestinal contents contained 4476 mg. of reducing substances, of which 3530 mg. were unfermentable. 20 cc. of urine gave no qualitative reduction test.

† Animal killed. Intestinal contents contained 3172 mg. of reducing substances, of which 3163 mg. were unfermentable. 5 cc. of urine gave a very faint reduction test.

galactose and lactose tending to support this view. The appearance of galactose in the urine following the ingestion of lactose would presumably be due to hydrolysis in the gut.

Since tartrate nephritis with practically complete suppression of the urine was without striking influence on the rate of removal from the blood of intravenously injected galactose (Corley, 1927), it is felt that excretion is not a necessary or essential part of the disposal of this sugar. The tissues must be able to metabolize

the galactose if sufficient time is available, excretion occurring only if the rate of removal leaves enough in the blood to exceed the renal threshold. Lactose in the tissues past the intestinal threshold is, like xylose, a very difficultly metabolized sugar, the failure of urinary excretion being associated with a decreased rate of removal from the circulation. That this effect is dependent upon changes in excretory ability rather than upon a more fundamental influence on metabolism, may be assumed since the rate of removal of circulating lactose was but slightly delayed when tartrate poisoning did not prevent urinary excretion of the substance (Rabbit 27, Table I).

Although lactose has been reported to relieve insulin intoxication when taken by mouth (Voegtlin et al., 1925; Moschini, 1924), it has been found lacking in beneficial effects when administered parenterally (Herring et al., 1924; Noble and Macleod, 1923).

Blanco (1928) has recently reported results of studies on the amounts of a number of sugars in the blood after administration. His method was not dissimilar to the one used in the present series of studies, as it is based on the lack of ability of yeast to remove sugars, particularly the unfermentable ones, from solution. In reporting his results he has corrected his values for residual reduction of the blood, and tabulated the differences as the sugar in question, whereas we have preferred to avoid this procedure. With this distinction in mind, it will be observed that while there is a certain amount of accord between the two groups of results, there are also certain obvious disagreements. It may be questioned however, what significance is to be attached to changes in the glucose of the blood when non-fasting animals are employed. The same consideration should not be disregarded when results obtained with galactose are interpreted (cf. Corley, 1928).

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AMINO ACID CATABOLISM.

II. THE FATE OF β-ALANINE AND ε-AMINOCAPROIC ACID IN THE PHLORHIZINIZED DOG.*

By RALPH C. CORLEY.

(From the Department of Bio-Chemistry of the School of Medicine, Tulane University, New Orleans.)

In a former communication (Corley, 1926) evidence was presented that γ-aminobutyric acid is, while δ-aminovaleric acid is not a sugar former in the phlorhizinized dog. Since ornithine yields extra glucose in the diabetic organism while lysine does not, the suggestion was offered that one of the paths of catabolism of the diamino acids is through the stage of the acids having 1 less carbon atom and with an amino group in the terminal position. In addition it was suggested that one of the paths of breakdown of these latter compounds may be through the corresponding dicarboxylic acids.

It has seemed of interest to continue the observations to several other ω-amino acids. The present is a report of the extra sugar formation in the completely phlorhizinized dog following the administration of β-alanine and ε-aminocaproic acid. These compounds were obtained from the Department of Chemistry of the University of Illinois, through the kindness of Dr. C. S. Marvel, to whom I wish to express my very sincere appreciation. The β-alanine contained 15.96 per cent of nitrogen (theoretical 15.73 per cent) and had a melting point of 194–196° (generally accepted 196°). The ε-aminocaproic acid contained 10.50 per cent of nitrogen (theoretical 10.69 per cent) and had a melting point of 201–202° (generally accepted 202–203°).

Dogs fasted for several days were treated with purified phlor-

hizin until the G:N ratio had achieved a satisfactory constancy. Thereupon the compounds studied were administered subcutaneously in aqueous solution in comparatively small individual doses, the first immediately subsequent to the collection by catheterization of the final portion of the urine of the preceding experimental day.   In the calculation of the extra glucose, there has been subtracted from the net G:N ratio (*i.e.* the ratio obtained after correcting for the nitrogen in the compound injected) of the day the acid was administered, the average of the values of the preceding day and of the following day, and the resulting figure has been multiplied by the nitrogen excretion for the ex-

<center>TABLE I.</center>
<center>*Sugar Formation from β-Alanine.*</center>

Dog 8.

Day of fasting.	Weight.	Urinary glucose.	Urinary nitrogen.	G:N
	kg.	*gm.*	*gm.*	
4	9 40	24 46	8 27	2 95
5	9 15	23 90	7 90	3 03
6	8 85	24 16	7 31	3 30
7	8 50	23 90	7.73	3 09
8	8 30	16 36	4 76	3.43

1.3 gm. of phlorhizin on the 2nd day; 1.0 gm. of phlorhizin from the 3rd day.   5.5 gm. of β-alanine subcutaneously in 20 per cent aqueous solution in three doses at 9.55 a.m., 2.35 p.m., and 5.35 p.m., on the 7th day.   Nonprotein nitrogen of blood 34.2 mg. per cent on preceding day; 31.4 mg. per cent on following day.   Extra glucose 1.12 gm.

perimental day.   The non-protein nitrogen of the blood has been determined prior to, and the day following the administration of the compound studied, to check the possibility that renal injury might cause nitrogen retention, thus invalidating the significance of the results obtained.   On this basis, ε-aminocaproic acid manifested a slight, β-alanine a rather decided nephropathic effect in but one dog each.   Two more satisfactory experiments in addition have been conducted for each compound.   Table I presents the results obtained in one study with β-alanine.   Two protocols for ε-aminocaproic acid are presented (Table II) since the results of these are not in strict accord.

In three experiments, 17.5 gm. of β-alanine, which would yield

11.81 or 17.7 gm. of extra sugar if 2 or 3 carbon atoms respectively were converted to glucose, caused an apparent sugar excretion of 4.5 gm. If however there is excluded from consideration the results of one experiment in which there was very apparent nitrogen retention, the administration of 11.5 gm. of β-alanine was followed by an extra elimination of 1.12 gm. Since this is a value that is comparatively negligible, it is felt that the figures ob-

TABLE II.

Sugar Formation from ε-Aminocaproic Acid.

Day of fasting.	Dog 5.				Dog 9.			
	Weight.	Urinary glucose	Urinary nitrogen	G:N	Weight.	Urinary glucose.	Urinary nitrogen.	G:N
	kg.	*gm.*	*gm.*		*kg.*	*gm.*	*gm.*	
3					7 55	23 90	7 50	3 19
4	11 80	40 60	11 34	3 58	7.15	24 94	7 38	3 37
5	11 30	38 52	9 72	3 96	6 78	26 50	7 45	3 56
6	10 85	35 84	11 09	3 23	6 65	18 64	6 55	2 84
7	10 30	33 78	10 00	3 38	6 35	14 50	4 06	3 57
8	10 00	30 40	9 60	3 17				
9	9 80	29 36	9 07	3 23				

Dog 5.—1.2 gm. of phlorhizin daily from the 2nd day. 9 gm. of ε-aminocaproic acid in 20 per cent aqueous solution subcutaneously in three doses at 9.45 a.m , 1.30 p.m., and 4.30 p.m. on the 7th day. Non-protein nitrogen of blood 45.0 mg. per cent previous day; 43.5 mg. per cent following day. Extra glucose 4.79 gm.

Dog 9.—1 gm. of phlorhizin daily from the 2nd day. 9 gm. of ε-aminocaproic acid in 20 per cent aqueous solution subcutaneously in three doses at 9.45 a.m., 2.30 p.m., and 5.30 p.m. on the 6th day. Non-protein nitrogen of blood 35.0 mg. per cent previous day; 37.5 mg. per cent following day. Extra glucose, none.

tained warrant the conclusion that β-alanine does not form glucose in the phlorhizinized dog.

When administered to three dogs, 27 gm. of ε-aminocaproic acid, which would furnish 12.36 or 18.54 gm. of additional glucose if 2 or 3 carbon atoms respectively were so converted, caused the excretion of 7.99 gm. of sugar. With two of the dogs (9 gm. of ε-aminocaproic acid, with theoretical glucose equivalents of 4.12 or 6.18 gm. if 2 or 3 carbons were converted) the actual sugar elimination was found to be 3.20 and 4.79 gm. respectively, the

one value considerably below the calculated amount for 2 carbon atoms, the other above this but below that for 3 carbon atoms. In consideration of these facts and the finding in one experiment of no neogenetic glucose, while there is unquestionably room for doubt, it is felt that it is unwarranted to conclude that ε-aminocaproic acid is a sugar former in the diabetic organism.   In fact it is probably better to take the results as definitely showing that this compound does not yield glucose in the phlorhizinized dog.

<center>DISCUSSION.</center>

Negative results in experiments such as these are not so readily applied in a consideration of the fate of the compounds studied as are positive findings.   Reductive deamination and α-oxidation may be considered as unlikely in the catabolism of β-alanine since they would yield respectively propionic acid and glycocoll, sugar formers.   A decision that β-oxidation occurs is difficult because of the lack of conclusive evidence as to the fate of malonic acid in the animal organism.

The observations that substituted ω-amino acids may be broken down 2 carbon atoms at a time (Peters, 1926; Thomas and Schotte, 1919), are of questionable significance for the interpretation of the manner of breakdown of the ω-amino acids themselves, since such alterations in the molecule would unquestionably be accompanied by considerable changes in property.

Thomas and Goerne (1914) found that ε-aminocaproic acid partially escaped utilization and appeared in the urine after it was fed to rabbits.   Reductive deamination, α-oxidation, γ-oxidation, or δ-oxidation of ε-aminocaproic acid would yield the non-sugar formers, caproic acid, δ-aminovaleric acid, β-aminopropionic acid, or glutaric acid but probably these can be ruled out as possible intermediates since analogous reactions (*i.e.* with similar relationships to either the carboxyl or amino groups) cannot be accepted for the two next lower homologues.   Although Thomas and Goerne (1914) made such a suggestion at one time, β-oxidation to yield γ-aminobutyric acid would appear unlikely in view of the definite gluconeogenesis from the latter.   Oxidation of the terminal carbon atom would yield adipic acid, about the mechanism of the catabolism of which little is known.   It is not readily attacked in the body since, when administered, a large

portion may be recovered in the urine.   β-Oxidation would yield succinic acid, and it is conceivable but rather improbable that the present not entirely concordant results with ε-aminocaproic acid might be explained as due to sugar formation from such fraction of the adipic acid as was further metabolized.

Keil (1927) found that after injecting 40 gm. of δ-aminovaleric acid into a dog, it was possible to isolate from the urine a small amount of amino ethyl methyl ketone, produced presumably by β-oxidation and subsequent decarboxylation.   The finding that γ-aminobutyric acid yielded 3 of its carbons as glucose (Corley, 1926), is however not readily explainable on the basis of β-oxidation.   Several possibilities come to mind in this regard, but in the absence of information about certain conceivable intermediates, the question may be left open for the time being.

SUMMARY.

The results obtained with the ω-amino acids, β-alanine, and ε-aminocaproic acid have been interpreted to indicate that these substances are not sugar formers in the completely phlorhizinized dog.

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BLOOD AS A PHYSICOCHEMICAL SYSTEM.

VIII. DIABETIC COMA.

By D. B. DILL, A. V. BOCK, J. S. LAWRENCE, J. H. TALBOTT, AND
L. J. HENDERSON.

*(From the Medical Laboratories of the Massachusetts General Hospital and the
Fatigue Laboratory, Morgan Hall, Harvard University, Boston.)*

A preliminary study of the physicochemical properties of blood
in diabetic coma has been presented by L. J. Henderson (1).
Recently a second case has been investigated and we are now able
to describe more precisely the properties of blood in this condition.

The data obtained for construction of carbonic acid dissociation
curves of arterial and of venous blood are shown in Table I. It
turns out that the height and slope of these curves are approxi-
mately the same for our two cases. There are some irregularities
in the relationship of oxygenated and of reduced blood in the case
of T.F.B. On the other hand all but one of the twelve deter-
minations of total carbon dioxide content of blood and plasma in
the case of M. D. are consistent as judged by comparison in Fig. 1
of the carbonic acid dissociation curves of his blood with similar
curves for a normal individual. In each case, a family of nearly
straight lines defines the relation of log pCO_2 to log total CO_2.
Similar information with regard to oxygen dissociation curves is
given in Table II and Fig. 2. Observations on serum and cell
chloride of equilibrated blood for each case are shown in Table
III. Miscellaneous observations are collected in Table IV.

The values for pH, given in Table III, calculated in the usual
manner from the carbonic acid dissociation curves of true plasma,
are probably within $\pm$ 0.03 of the true values. The value of 6.12
for pK', cannot be far wrong even in diabetic coma since Cullen,
Keeler, and Robinson (2), and more recently Hastings, Sendroy,
and Van Slyke (3), have found that in pathological sera the value
for pK', varies slightly with variation of pathological state, salt

content, protein, or pH itself.   However, there is some uncertainty regarding concentration of free carbonic acid in the serum. It has been shown by Van Slyke, Hastings, Sendroy, and Neill (4) that the solubility of carbonic acid in lipemic serum may be 7 or 8 per cent greater than in normal serum.   In view of the heterogeneity of lipemic serum it is not clear that the increased propor-

TABLE I.

Carbon Dioxide Dissociation Curves in Diabetic Coma.

Subject.	Blood used.	pCO_2	pO_2	Total CO_2 content.	
				Whole blood.	True plasma.
		mm. Hg	*mm. Hg*	*vol. per cent*	*vol. per cent*
T. F. B.	Venous.	4.1	Air.	5.08	4.60
"	"	14 0	"	8.85	8 35
"	"	59.9	"	$\begin{cases} 22.90 \\ 22.12 \end{cases}$	23.00
"	"	3.4	13.0	4.50	4.00
"	"	61.5	11.6	24.23	24.80
"	Arterial.	8.6	Air.	$\begin{cases} 6.52 \\ 7.03 \end{cases}$	
"	"	20.0	"	10.39	
M.D.	Venous.	4.4	"	4.04	4.99
"	"	12.9	"	7.99	
"	"	41.9	"	17.20	17.20
"	"	208.0	"	47.20	49.50
"	"	0.5	2.6	2.84	
"	"	10.1	2.2	7.61	
"	"	40.5	2.5	18.66	
"	"	208.6	2.2	50.60	
"	Arterial.	9.7	Air.	6.50	

tion of free carbonic acid (due to its relatively high solubility in fat globules) should be taken into account in estimating pH_s.

In Fig. 3 observed cell volume in the case of T.F.B. is plotted as a function of calculated pH_s.   It appears from this figure that an error was made in determining the cell volume in one tube for one point is widely separated from the other three and indicates a decrease in cell volume with increase in acidity.   Since all our previous work shows that this is impossible on the alkaline side of the isoelectric point of hemoglobin, the point has been discarded.
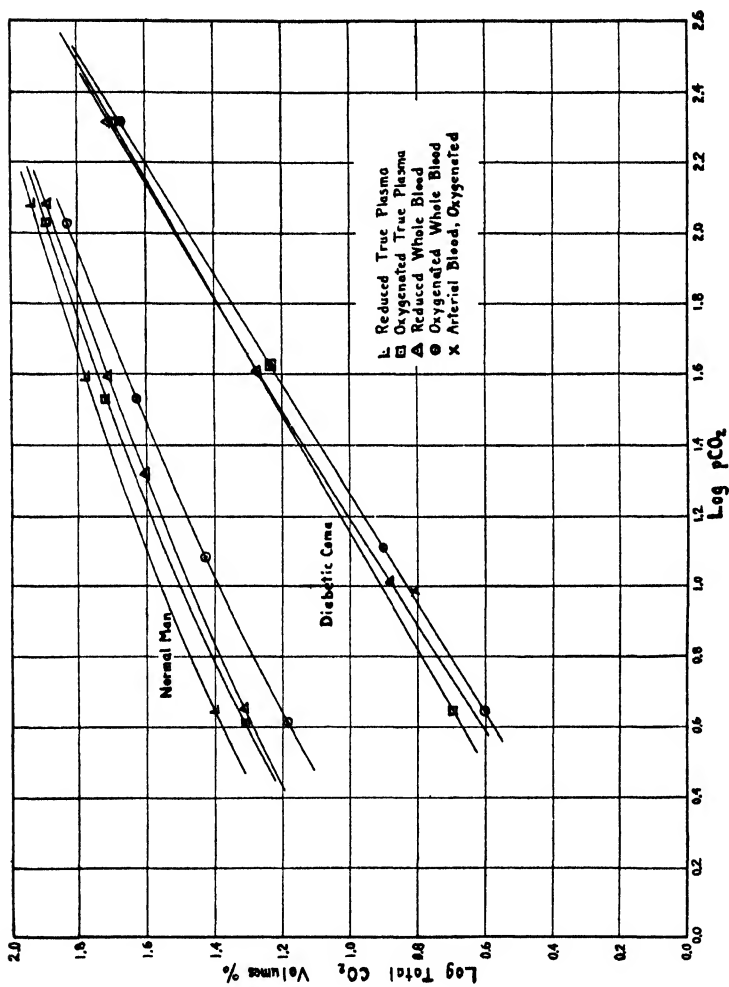
Fig. 1. Carbonic acid dissociation curves in diabetic coma (Case M.D.) compared with similar curves for normal man.
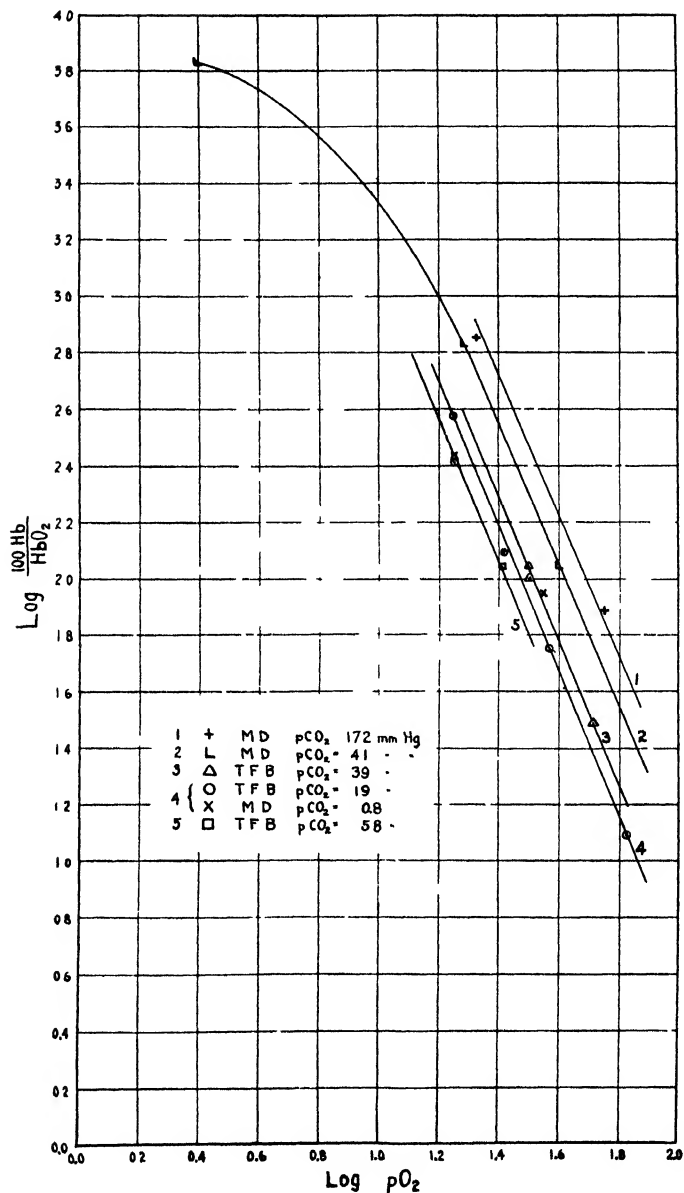
Fig. 2. Oxygen dissociation curves in diabetic coma.

TABLE II.

Oxygen Dissociation Curves in Diabetic Coma.

Oxygen capacity of venous blood used = 22.0 volumes per cent in each case.

Subject.	pCO_2	pO_2	Total O_2 content.	HbO_2 content.	O_2 saturation.
	mm. Hg	*mm. Hg*	*vol. per cent*	*vol. per cent*	*per cent*
T. F. B.	5.5	18.0	6.12	6.07	27.6
"	6.0	26.0	10.50	10.44	47.4
"	19.1	17.7	4.62	4.58	20.8
"	17.5	26.3	9.87	9.81	44.6
"	19.7	37.2	14.13	14.04	63.8
"	19.2	67.2	19.75	19.58	89.0
"	38.2	31.8	{10.49	10.41	49.9
			11.06	11.98	47.3
"	39.9	52.2	16.91	16.78	76.3
M. D.	0.9	18.0	5.94	5.90	26.8
"	0.7	35.5	11.79	11 70	53.1
"	41.4	19.3	2.87	2.82	12.8
"	42.0	39.9	10.50	10.40	47.2
"	40.5	2.5	0.32	0.32	1.45
"	170.5	21.3	2.80	2.74	12 4
"	174.5	57.4	12.62	12.47	56.7

TABLE III.

Chloride Distribution in Diabetic Coma.

Subject............	T. F. B.				M. D.	
Tonometer....................	282	275	265	335	305	279
HbO_2, *per cent*.................	100	100	17	9	100	100
Serum Cl, *mM per l. serum*.......	100.7	103.5	106.5	99 9	100 8	97 2
Cell " " " " cells.........	67.8	70.2	68 8	75.1	69 9	78.3
" volume, *per cent*............	49.6	49.3	49.5	50 45	51.0	52 0
" " corrected, *per cent*..		50.3				
Serum H_2O, *cc. per l. blood*......	466	458	467	457.5	453	443
Cell " " " " "	353	361	352	361.5	378	388
Serum Cl, *mM per l. blood*........	(50.8)	51.35	53.8	49.5	49.4	46.7
Cell " " " " "	33.6	35.35	34.1	37.9	35.7	40 7
Blood " " " " "	(84.4)	86.7	87.9	87.4	85.1	87.4
r_{Cl}.............................	0.83*	0.88	0.84	0.98	0.87	1.00
pH_s.............................	7.31	6 76	7.37	6.81	7.32	6.53

* This value for r_{Cl} is estimated from the average value for blood Cl, 87.3, and the determined value for cell Cl, 33.6. The determined value for serum Cl, 50.8, is obviously incorrect.

In its place an estimated value, read off the curve of Fig. 3, has been employed in calculating chloride distribution.   On this same figure the relation of cell volume to pH_s is shown for both oxygenated and reduced blood calculated from the equations of Van Slyke, Wu, and McLean (5) as extended by one of us (6).   It will be noted that the rate of change of cell volume with pH_s is much less
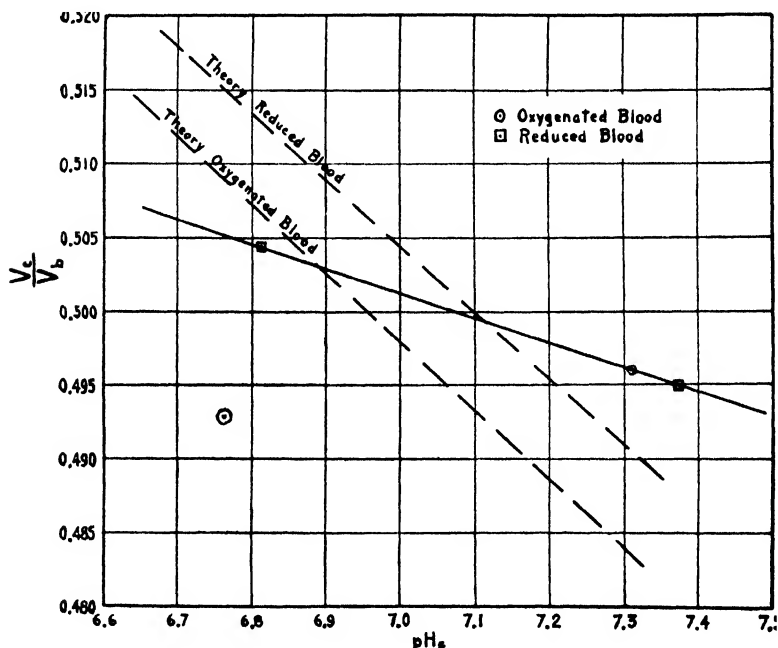
Fig. 3. Cell volume as a function of pH_s (Case T. F. B.).

in T. F. B. than that implied by the theory of Van Slyke. A similar observation was made in the other case of diabetic coma. From the calculated curve, $\dfrac{d\left(\dfrac{V_c}{V_b}\right)}{dpH_s} = 0.045$ while direct determination of cell volume changes indicates a maximum value of only 0.016.   This discrepancy will be discussed later; for reasons there given the experimental value of 0.016 is employed in constructing the nomograms.

Determination of water was made gravimetrically on one sample each of serum and cells as shown in Table IV. Changes in serum and cell water were calculated from volume changes with the assumption that small changes in volume are accompanied by equal changes in water. Values for cell water and serum water, thus calculated for equilibrated blood, and for cell volume are found in Table III. Values for r_{Cl} are given for each case in this table. With the data given in Tables I and III, r_{HCO_3} can be calculated also. In each case of diabetic coma, values for r_{Cl}

TABLE IV.

Miscellaneous Observations in Diabetic Coma.

	T. F. B.	M. D.
HbO$_2$ capacity, venous blood, *vol. per cent*.....	22.0	22.0
" " arterial " " " ".....	22.05	22.4
" content, " " " " "....	21.29	
" per cent in arterial blood,...............	96.5	
Total CO$_2$ content, arterial blood, *vol. per cent.*	6.22	5.54
" " " venous " " " "		9.92
Serum protein (protein nitrogen $\times$ 6.25), *gm. per l. serum*.......................................	65.0	83.3
Serum base, *mM per l. serum*....................	151.2	150.0
Cell " " " " cells.....................		100.5
Serum Na + K,* *mM per l. serum*.............		140.6
Cell " + " " " " " "		94.4
Serum water, *cc. per l. serum*..................	924 0	921.2
Cell " " " " cells..................	712 2	744 0

* The content of sodium + potassium in serum and in cells was determined by Stoddard's method (Stoddard, J. L., *J. Biol. Chem.*, **74**, 677, (1927)).

exceeded the normal value, for a given pH, value, by about 0.2. Under the conditions of our experiments r_{Cl} can be determined with a much higher degree of accuracy than r_{HCO_3}. In the case of T. F. B., r_{HCO_3} exceeds unity while in the other case the values found for the physiological range were not far from normal. If we adopt the relation, $r_{Cl} = 0.87r_{HCO_3}$, established for human blood by Hastings, Sendroy, McIntosh, and Van Slyke (7), r_{HCO_3} would approximate 1.0 in the physiological range. Since the procedure for determining r_{HCO_3} is rather inaccurate in acidosis,

FIG. 4. Blood of T. F. B.

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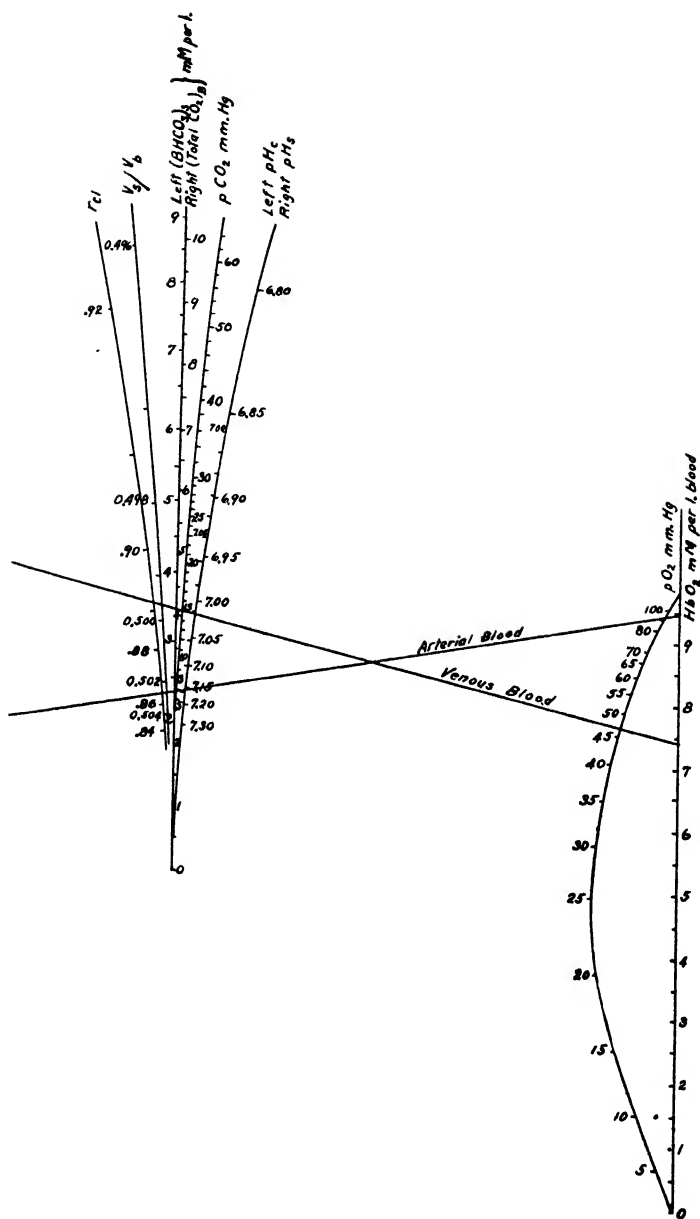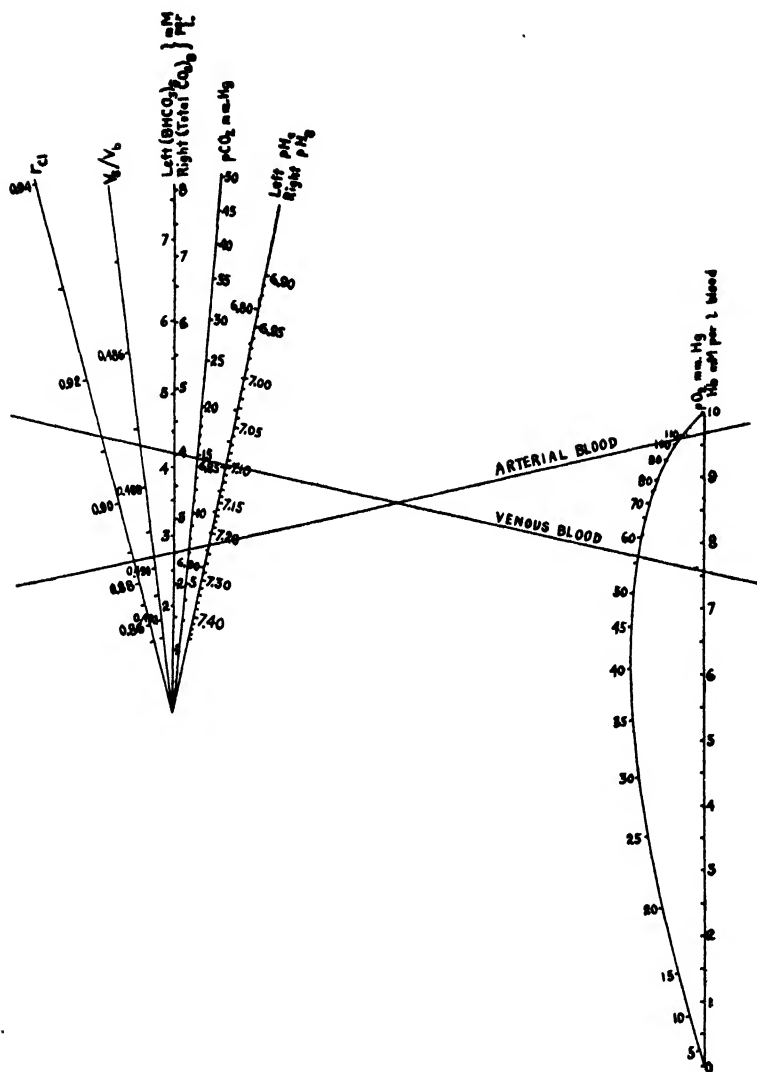
Fig. 5. Blood of M. D.

TABLE V.

Blood in Diabetic Coma.   Case T. F. B.

Concentration of hemoglobin   =   9.82 mM per liter of blood.

" serum proteins = 32.7 gm. " " " "

Respiratory quotient   =   0.69

	Arterial			Venous			Δ		
	Serum.	Cells.	Whole blood.	Serum.	Cells.	Whole blood.	Serum.	Cells.	Whole blood.
H_2O cc. per l. blood.........	464.7	354.3	819.0	461.5	357.5	819.0	−3.2	+3.2	0.0
B mM " " "	76.00	58.92	134.92	76.00	58.92	134.92	0.0	0.0	0.0
X " " " "	16.94	−1.69	15.25	17.71	−2.22	26.33	+0.77	−0.53	0.24
Cl " " " "	52.62	34.66	87.28	51.59	35.69	87.28	−1.03	+1.03	0.0
BP " " " "	5.26	24.53	29.79	4.92	23.41	28.33	−0.34	−1.12	−1.46
$BHCO_3$ " " " "	1.18	1.42	2.60	1.78	2.04	3.82	+0.60	+0.62	+1.22
H_2CO_3 " " " "	0.10	0.08	0.18	0.23	0.18	0.41	+0.13	+0.10	+0.23
Total CO_2 " " " "	1.28	1.50	2.78	2.01	2.22	4.23	+0.73	+0.72	+1.45
Free O_2 " " " "			0.10			0.05			−0.05
Combined O_2, mm. Hg.........			9.48			7.44			−2.04
Total O_2 " "			9.58			7.49			−2.09
pCO_2, mm. Hg.........			6.6			15.6			+9.0
pO_2, " "			92.0			47.0			−45.0
Volume, cc. per l. blood.........	502.7	497.3	1000.0	499.5	500.5	1000.0	−3.2	+3.2	0.0
pH.........	7.165	7.110		7.015	7.078		−0.150	−0.032	
rCl.........			0.864			0.893			+0.029
rHCO₃.........			1.58			1.48			−0.10

TABLE VI.

Blood in Diabetic Coma. Case M.D.

Concentration of hemoglobin = 10.0 mM per liter of blood.

" serum proteins = 42.6 gm. " " "

Respiratory quotient = 0.70

	Arterial.			Venous.			Δ		
	Serum.	Cells.	Whole blood.	Serum.	Cells.	Whole blood.	Serum.	Cells.	Whole blood.
H₂O cc. per l. blood	451.1	379.9	831.0	448.7	382.3	831.0	−2.0	+2.0	0.0
B mM " " "	73.45	50.0	122.55	73.45	50.0	122.55	0.0	0.0	0.0
X " " " "	15.69	−3.74	11.95	16.18	−4.23	11.95	+0.49	−0.49	0.0
Cl " " " "	49.40	36.80	86.20	48.56	37.64	86.20	−0.84	−0.84	0.0
BP " " " "	7.02	16.00	23.02	6.66	15.06	21.72	−0.36	−0.94	−1.30
BHCO₃ " " " "	1.34	0.94	2.28	2.05	1.53	3.58	+0.71	+0.59	+1.30
H₂CO₃ " " " "	0.11	0.10	0.21	0.22	0.20	0.42	+0.11	+0.10	+0.21
Total CO₂ " " " "	1.45	1.04	2.49	2.27	1.73	4.00	+0.82	+0.69	+1.51
Free O₂ " " " "			0.12			0.06			−0.06
Combined O₂ " " " "			9.65			7.56			−2.09
Total O₂ " " " "			9.77			7.62			−2.15
pCO₂, mm. Hg			7.2			14.7			+7.5
pO₂, " "			105.0			57.0			−48.0
Volume, cc. per l. blood	489.7	510.3	1000.0	487.3	512.7	1000.0	−2.0	+2.0	0.0
pH	7.222	6.887		7.097	6.848		−0.125	−0.039	
rCl			0.887			0.912			+0.025
rHCO₃			0.83			0.88			+0.05

particularly in the alkaline range, we must remain somewhat un-
certain regarding the true value of this variable in diabetic coma.

With the data thus treated, the nomograms, Fig. 4 for T. F. B.
and Fig. 5 for M. D., were constructed in the usual manner.
From them and from data given in Table IV, were derived Tables
V and VI showing changes in the blood in the respiratory cycle
and Tables VII and VIII showing the composition of arterial

TABLE VII.

Arterial Serum in Diabetic Coma* and in Normal Man.

					Diabetic coma.		Normal man.†
					T. F. B.	M. D.	
H_2O	*cc.*	*per*	*l.*	*serum*...............	924.0	921.2	943.3
B	*mM*	"	"	"	151.2	150 0	154.0
X	"	"	"	"	33.70	32 0	17.0
Cl	"	"	"	"	104.7	100.9	99 32
BP	"	"	"	"	10.45	14 36	13.13
$BHCO_3$	"	"	"	"	2.35	2.74	24.55
H_2CO_3	"	"	"	"	0.20	0 22	1.22
Total CO_2	"	"	"	"	2.55	2 96	25.77
pH..					7.165	7.222	7.425
Protein, *gm. per l. serum*..................					65 0	87.1	72.5

* The values given are for serum approximately free from fat.   The value
for protein given for T. F. B. may be too low, for the value of 924 serum
water implies protein content higher than normal.

† The values given for normal man in Tables VII and VIII are for A. V.
B. at rest (Bock, A. V., Dill, D. B., Hurxthal, L. M., Lawrence, J. S., Cool-
idge, T. C., Dailey, M. E., and Henderson, L. J., *J. Biol. Chem.*, **73**, 749
(1927)).

serum and of arterial cells in comparison with similar observations
on normal man.   The pH_c values shown in the nomograms and
tables were derived in a special manner which will be discussed
below.

The position of the arterial line on the nomogram is fixed by
direct examination of arterial blood.   The position assigned to
the venous line is based upon knowledge of the respiratory quo-
tient in diabetic coma as reported by Joslin (8) together with a
guess as to rate of blood flow.   Besides high pulse rate, we have
two clues as to rate of blood flow.   On the one hand in the case
of T. F. B. the value for oxygen saturation in arterial blood (96.5

per cent) implies an oxygen pressure in arterial blood of 92 mm., a normal value. However, the alveolar oxygen pressure must have been not less than 130 mm. This high value is deduced from the facts: (a) For alveolar air, in general, $pO_2 + pCO_2 = 150 \pm 10$ and (b) examination of the arterial blood indicated that air in equilibrium with it should have a pCO_2 value of from 6 to 7 mm. With such a pressure head of oxygen and 96.5 per cent saturation the rate of blood flow was possibly faster than normal and almost certainly not slower than normal. On the other hand, in recent experiments now in course of publication (9) rate of blood flow was determined by the method of Field, Bock, Gildea, and Lathrop

TABLE VIII.

Arterial Cells in Diabetic Coma and in Normal Man.

					Diabetic coma.		Normal man.
					T. F. B.	M. D.	
H_2O	cc	per	l.	cells..............	712 2	744 0	705 0
B	m_M	"	"	"	118 50	98 0	133 75
X	"	"	"	"	−3 34	−7 26	17 73
Cl	"	"	"	"	69 66	72 10	45 27
BP	"	"	"	"	49 33	31 32	56 50
$BHCO_3$	"	"	"	"	2 85	1 84	14 25
H_2CO_3	"	"	"	"	0 16	0 20	0 93
Total CO_2	"	"	"	"	3 01	2 04	15 18
Combined O_2	"	"	"	" ....,	19 06	18 90	21 43
Total Hb	"	"	"	"	19 75	19 58	22 33
pH................................					7 165	6 887	7 124

(10), in ammonium chloride acidosis in which carbonic acid capacity was lowered to one-half its normal value. Rate of blood flow remained normal or increased not more than one-fifth.

With the nomograms at hand we may return to a discussion of the discrepancy mentioned above, *viz.* that $\dfrac{d\left(\dfrac{V_c}{V_b}\right)}{dpH_s}$ has a much lower value than that required by the approximation of Van Slyke, Wu, and McLean (5). If this difference is real and not due to experimental error it seems probable that $\dfrac{dpH_c}{dpH_s}$ in these experiments has a value much smaller than normal. Here we are again

faced with inadequate experimental data.   The remarks which have been made regarding the accuracy of r_{HCO_3} calculations apply also to pH_c calculations.   There is the additional fact that the value 5.93 for pK'_c was fixed by Van Slyke, Hastings, Murray, and Sendroy (11) for cells of horse blood and its applicability to pathological conditions in man has never been determined.   From these carbonic acid dissociation curves it is clearly impossible to make an accurate calculation of $\dfrac{dpH_c}{dpH_s}$ or anything more than a rough estimate of the magnitude of pH_c.

But in the oxygen dissociation curves of Fig. 2, we have information from which $\dfrac{dpH_c}{dpH_s}$ can be calculated.   Theories of oxygen dissociation curves have been reviewed by Adair (12) who also has proposed a theory of his own.   He found, under the conditions of his experiments, with hemoglobin prepared from blood of normal man, that *"pH practically determines the affinity of hemoglobin for oxygen."*   However, it is certain that in pathological human blood pH_c, as determined from the carbonic acid dissociation curves, assuming $pK'_c = 5.93$, does not determine the absolute position of the oxygen dissociation curve.   This has been shown by Henderson (1) who has deduced empirically a roughly approximate relation between $f(H^+)_c$ and $\log pO_2$ where $f(H^+)_c = \log \dfrac{1 + 10^{8.18} (H^+)_c}{1 + 10^{6.62} (H^+)_c}$ and pO_2 the pressure of oxygen when $HbO_2 = 50$ per cent.

A study of the position (as defined by this value of pO_2) of oxygen dissociation curves in diabetic coma reveals a different condition than that in normal man.   In Fig. 6 we have plotted pO_2 at one-half saturation as a function of pH_s.   These variables are known quite accurately and there is no doubt about the reality of the differences found.   It is evident that the two cases of diabetic coma, near the physiological range, show small change in position of oxygen dissociation curves with large change in pH_s. From $pH_s = 7.2$ to $pH_s = 7.3$, values for $\dfrac{\Delta pO_2}{\Delta pH_s}$ are respectively, T. F. B., 3; M. D., 10; A. V. B. (normal), 43; Gladys B. (normal), 35.   We must conclude that (a) the position of the oxygen disso-
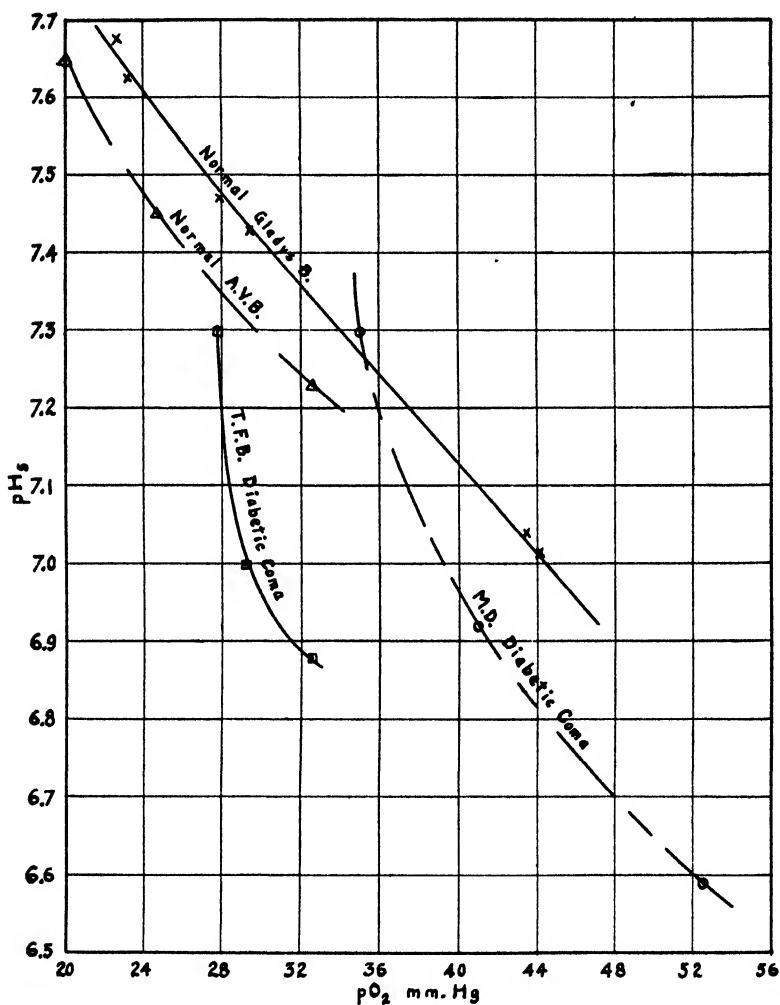
Fig. 6. Position of oxygen dissociation curves (pO_2 when $HbO_2 = 50$ per cent) as a function of pH_s. Normal subjects compared with diabetic coma.

ciation curve in human blood is not determined by pH_s; (*b*) change in position of the oxygen dissociation curve is not a simple function of pH_s; (*c*) either $\dfrac{dpH_c}{dpH_s}$ in diabetic coma has an abnormally
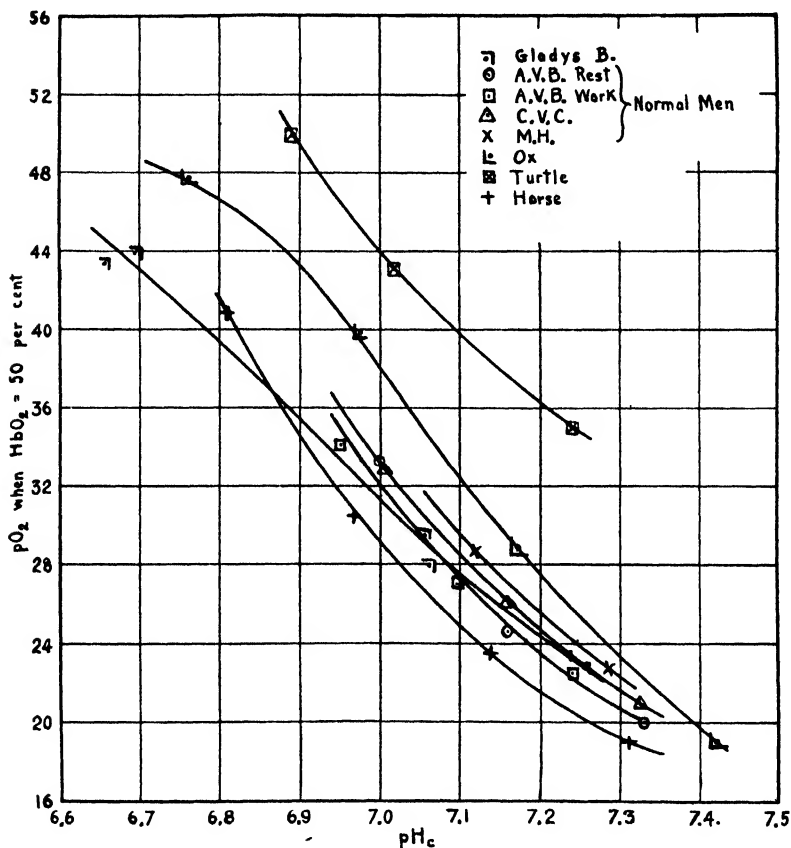
FIG. 7. Position of oxygen dissociation curves as a function of pH_s.

small value or else change in position of the oxygen dissociation curve is not a simple function of pH_c.

Even though it is impossible to predict accurately the position of a single oxygen dissociation curve in a specimen of blood whose carbonic acid dissociation curves are known, it is likely that in

human blood near the physiological range a given small change in the position of an oxygen dissociation curve corresponds to an approximately constant increment in pH_c. We thus tentatively accept as more probable the former of the two alternatives mentioned above. In order to make use of this hypothesis we have plotted in Fig. 7 as a function of pH_c, calculated from the carbonic acid dissociation curves, the position of the oxygen dissociation curves of three normal men, a normal woman, the ox, the horse, and the turtle. It appears that parallel curves represent the relation between these two variables in normal human blood and somewhat less accurately in normal mammalian blood, in the physiological range. Thus values for $\dfrac{\Delta pO_2}{\Delta pH_c}$, between pH_c values of 7.1 and 7.2, are respectively: horse, 33; A. V. B., 37; C. V. C., 38; M. H., 39; Gladys B., 33; ox, 48; turtle, 35. We shall assume that a similar parallel curve holds for diabetic coma and later seek confirmation of this assumption from an independent source.

Table IX is derived from Table II and Figs. 2, 4, 5, and 7 in an obvious manner. As suggested above, we do not wish to attach importance to the absolute values of pH_c given in Column e of Table IX but rather to the rate of change of pH_c with pH_s. In normal blood between the pH_c range shown for T. F. B. in Table IX, $\dfrac{\Delta pH_c}{\Delta pH_s} = 0.80$ (13). In one case of diabetic coma (T. F. B.) this ratio has a value of 0.35 between pH_s values of 7.21 and 6.87. In the other case its value is 0.29 between pH_s values of 7.30 and 6.92.

It happens in the case of T. F. B. that when $HbO_2 = 50$ per cent the relation between pCO_2 and pH_c, as well as between pO_2 and pH_c, is approximately linear. The former relationship can be expressed by the equation

$$pH_c = 7.15 - 0.0036 \, pCO_2 \tag{1}$$

$$\text{or} \quad \frac{dpH_c}{dpCO_2} = -0.0036 \tag{2}$$

The absolute values given by Equation 1 are not important, but, as suggested above, Equation 2 probably holds approximately in this case. Given a base value for pH_c, Equation 2 can be used in
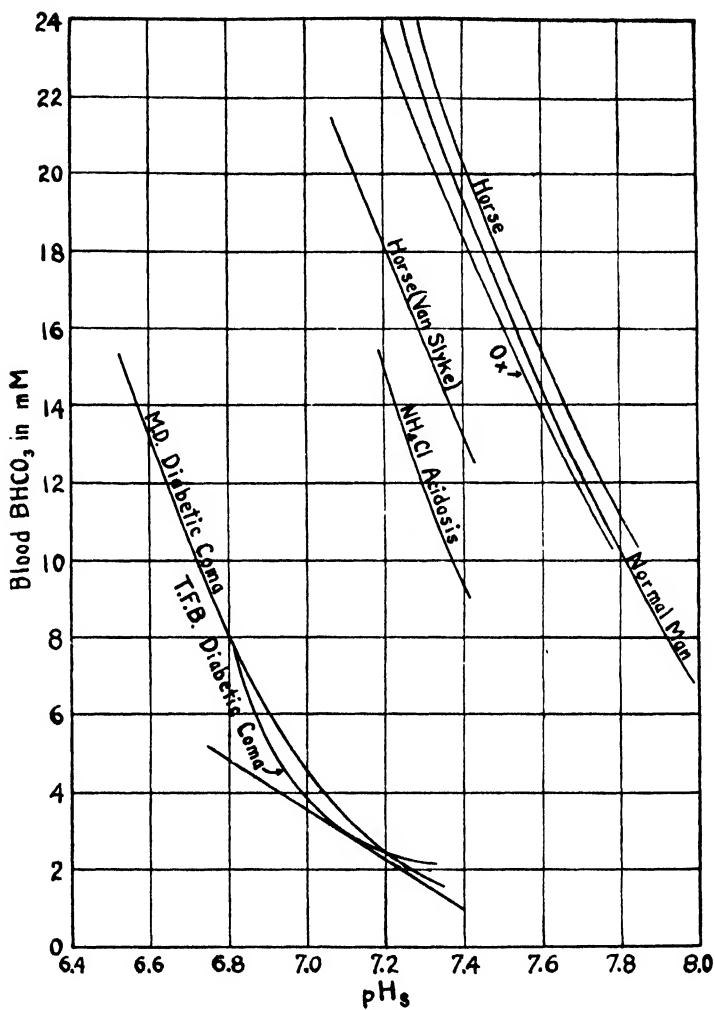
Fig. 8. Combined carbonic acid of oxygenated whole blood as a function of pH$_s$.

calculating other values for pH_c between the limits $pCO_2 = 5.8$ and $pCO_2 = 39$. The oxygen dissociation curves of M. D. cover a much wider range and as might be expected a relation similar to that shown above holds for only a portion of this range.

We may now consider other evidence to the effect that $\dfrac{dpH_c}{dpH_s}$ is much smaller than normal. In the respiratory cycle it appears probable that for whole blood $\Delta BHCO_3 = \Delta BP$. The relation between $\Delta(BHCO_3)_b$ and ΔpH_s in oxygenated horse blood has been studied by Van Slyke, Hastings, and Neill (14) and was found to be linear over the range covered. It is far from such in this case as is shown in Fig. 8 where comparison is made of the blood of the horse, ox, normal man, man in ammonium chloride acidosis, and man in diabetic coma. The data used in preparing Fig. 8, aside from those given in this paper, are being prepared for publication. In the physiological range of diabetic coma $\dfrac{d(BHCO_3)_b}{dpH_s} = -6.4$ in the case of T. F. B. and -11.0 in the case of M. D. as compared with the normal horse blood value of -23.7. This observation could have been predicted qualitatively from the nature of the case for a linear extrapolation of the approximately straight lines shown in Fig. 8 would intersect the line $(BHCO_3)_b = 0$ at pH_s values near 8.0. This we know to be impossible in blood of normal hemoglobin concentration. From the curves for diabetic coma shown in Fig. 8, it is clear that there must be a relatively great change in reaction of serum if carbonic acid is transported at the usual rate.

What does this imply with regard to the erythrocytes? If $\dfrac{dpH_c}{dpH_s}$ retains its normal value there must also be a great change in the reaction within the cell, the more so because here the effect of oxygenation on the acidity of hemoglobin is small. Van Slyke, Hastings, and Neill (14) found that in horse blood $\dfrac{d(BHCO_3)}{d(HbO_2)} = 0.55$ at $pH_s = 7.3$. In the two specimens of blood in diabetic coma, $\dfrac{d(BHCO_3)}{d(HbO_2)} < 0.15$ at $pH_s = 7.1$. Adair (15) found that on oxygenation ΔB rises from 0.06 at pH 6.6 to 0.51 at pH 7.4 and increases to 0.55 at pH 7.8.

As may be seen from the nomograms, Figs. 4 and 5, the respiratory cycle involves a change in pH_s of 0.15 in one case and 0.12 in the other.   A change in pH_c of any such magnitude as this would involve, according to the equations of Van Slyke, Wu, and McLean (5), change in $\Delta(BP)_c$ alone of about three times the magnitude of the observed variation in $\Delta(BHCO_3)_b$.   There is of course an alternative explanation; *viz.*, that the base-binding capacity of hemoglobin is much less than implied by the equation of Van Slyke, Wu, and McLean (5).[1]   However, the simplest method of reconciling differences is an assumption that $\dfrac{dpH_c}{dpH_s}$ is much smaller than normal.

We have therefore completed the pH_c line on the nomograms by fixing a value in the physiological range by calculation from carbon dioxide dissociation curves and estimating the rate of change of pH_c from the data of Table VII, *viz.* by the relationship of the oxygen dissociation curves one to another.   It will be noted that in the physiological range $\dfrac{\Delta pH_c}{\Delta pH_s}$ has a value of 0.22 in the case of T. F. B. (Table V) and 0.32 in M. D. (Table VI).   It turns out that with the values found for pH_c by this method of calculation, $\Delta BHCO_3$ approximately equals ΔBP when $(BP)_c$ is calculated by the equations of Van Slyke, Wu, and McLean (5).

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[1] There is quite good evidence that the formulas of Van Slyke, Wu, and McLean for horse blood do not give correct absolute values for base-binding capacity of cell protein in human blood.   Hastings, A. B., Sendroy, J., Jr., McIntosh, J. F., and Van Slyke, D. D., (*J. Biol. Chem.*, **79**, 193 (1928)) have pointed out that comparison of the data of Van Slyke, Wu, and McLean with those of Adair, G. S., (*J. Biol. Chem.*, **63**, 517 (1925)) indicates for the non-diffusible constituents of human cells at physiological pH_c ranges a much lower alkali-neutralizing capacity.   In our data, as shown in Table VIII, the sum $(P)'_c$ (calculated from pH_c by the formula of Van Slyke, Wu, and McLean) + $(Cl)'_c$ + $(HCO_3)'_c$ exceeds the value for $(B)_c$ in each case of coma.   In T. F. B., $(B)_c$ was determined by the empirical relation established by Van Slyke, Wu, and McLean, $(B)_c = 6.0(Hb)_c$.   In M. D. $(B)_c$ was determined experimentally as shown in Table IV.   However, while it may be that base-binding capacity is different, we have no new evidence as to rate of change in base-binding capacity with pH.

These observations and calculations are consistent with the observation that $\mathrm{d}\!\left(\dfrac{V}{V_b}\right)\Big/\overline{\mathrm{dpH}_s}$ has a value much less than normal.

Accordingly, experimental observations on this ratio have been employed rather than values calculated by the formulas of Van Slyke, Wu, and McLean (5).[2]

These results will be discussed from the clinical point of view at a later time. Also, in a succeeding paper of this series, a comparison will be made of blood in the acidosis of diabetic coma, in

TABLE IX.

Position of Oxygen Dissociation Curves and pH_s in Diabetic Coma.

Subject. (a)	pCO_2 from Table II. (b)	pO_2 when HbO_2 = 50 per cent from Fig. 2. (c)	pH_s from Figs. 4 and 5. (d)	pH_c estimated from Column c and Fig. 7. (e)
	mm. Hg	*mm. Hg*		
T. F. B.	5.8	26.8	7 21	7.11
"	19 0	29.2	6 975	7.055
"	39.0	32 5	6.87	6.99
M. D.	5.8*	35 0	7.30	6 95
"	41 0	41.0	6.92	6.84
"	172.0	52.5	6.59	?

* This value is obtained by interpolation.

ammonium chloride acidosis, in work acidosis, and in normal man. Little more remains to be added at this time except to refer very briefly to the comparisons made in Tables VII and VIII. The two cases of diabetic coma are similar in so far as serum is concerned but different in respect to composition of cells. There is certainly a much greater degree of acidosis in the cells in the case of M. D., as judged by the estimated carbonic acid content of cells as well as by the position of oxygen dissociation curves as shown in

[2] In this discussion the theories recently propounded by Henriques, O. M., (*Biochem. Z.*, **200**, 1. *et seq.* (1928)) have not been taken into consideration. It would not be difficult to interpret our findings in accordance with his theories. However, we are here concerned with an internally coherent explanation of the facts rather than with the underlying theories, a consideration of which may be deferred to a later date.

Table IX. We can only speculate as to the cause for this difference. The anomalous distribution of bromide ions between cells and plasma reported by Hastings and van Dyke (16) suggests that our observed differences in cell acidosis are related to difference in distribution of the new fatty anions. This might be related to difference in kind of new anions present in the two cases or to difference in permeability of cell membrane.

SUMMARY.

This paper illustrates the value of systematically studying the interrelation of the important variables in the blood, three at a time, and then seeking to coordinate the results into a coherent system. Facts which at first are bewildering and seem inexplicable can be fitted into a consistent pattern. Provided one is guided by certain established generalizations, chief among which is the mutual dependence of the important variables studied, it is possible in unforeseen ways to evaluate experimental observations, by discarding those which are inaccurate and using those which are accurate. Thus from a few experimental observations it has been possible in this instance to synthesize a physicochemical description of blood in diabetic coma, which we believe to be not far inferior to the best nomogram based upon full and accurate information.[3]

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13. Bock, A. V., Dill, D. B., Hurxthal, L. M., Lawrence, J. S., Coolidge, T. C., Dailey, M. E., and Henderson, L. J., *J. Biol. Chem.*, **73**, 749 (1927).

14. Van Slyke, D. D., Hastings, A. B., and Neill, J. M., *J. Biol. Chem.*, **54**, 507 (1922).

15. Adair, G. S., *J. Biol. Chem.*, **63**, 517 (1925).

16. Hastings, A. B., and van Dyke, H. B., *J. Biol. Chem.*, **78**, p. xxxv (1928).

Case Histories.

T. F. B., Case 285,994. — A man 24 years old, a leather worker, with a history of 3 months duration, characterized by polyuria, nocturia, polydipsia, a ravenous appetite, and a loss of 14 kilos in weight, entered the hospital September, 9, 1927, in coma of 12 hours duration. For the past months his bowels had been constipated. 7 days before entrance sugar had been found in his urine and a diet prescribed, without insulin. At this time also a furuncle was developing on the left side of his neck. 4 days before entrance he began to cough, raised greenish sputum, and noticed drowsiness. He was unable to work. The evening before entry he could be aroused only with difficulty.

On admission to the hospital he was in coma, with Kussmaul breathing, respiratory rate 30, pulse 112, temperature 96.2, blood pressure 118/50. His eyeballs were soft, the tongue parched, there was a draining furuncle at the base of the neck, and evidence of loss of weight. A few moist râles were present at the left base. The blood sugar was 260 mg. per 100 cc., non-protein nitrogen 34 mg., the urine showed albumin and positive tests for sugar and diacetic acid, and many casts were found in the sediment. The stomach was washed out and insulin was given at once subcutaneously and intravenously. Glucose and other fluids were given by mouth, by rectum, subcutaneously, and intravenously. After some hours, sodium bicarbonate, 14 gm., was given intravenously and small amounts given hourly by mouth. After 14 hours the patient was conscious, his urine sugar-free and acid-free, and the blood sugar 49 mg. per 100 cc. 480 units of insulin were given during the first 24 hours. The patient was discharged in good condition 19 days after admission on a diet of carbohydrate 40, protein 60, fat 140, and insulin given 15, 5, and 10 units before meals.

M. D., Case 287,333.—A Hebrew printer, age 37, entered the hospital on October 30, 1928, in diabetic coma of 3 or 4 hours duration. He had had a known history of diabetes for 9 years, had consulted many physicians, and

had had numerous periods of previous hospital care.   He had been unable to work since November, 1927.   In January, 1928, he had been treated for diabetic coma at a neighboring hospital.   Care with reference to diet had not been exercised.   Insulin had been given him morning and night, but the exact dosage was not known.   Constipation, great thirst, and frequency of urination had long characterized his illness.

7 days ago an infection began in the left temporal region, rapidly developing into a small carbuncle.   At 5 a.m. on the day of entry to the hospital he became nauseated, vomited, and complained of abdominal distension. The family physician referred him to the hospital.

On examination he was in coma, breathing stertorously with some tracheal râles.   He was emaciated and dehydrated.   The eyeballs were soft, the skin dry, pulse 132, rectal temperature 93.2°, blood pressure 120/80. There was a small, partially drained carbuncle on the left temple.   Both lungs presented coarse, moist râles.   The abdomen was slightly distended and tympanitic.   The blood sugar was 372 mg. per 100 cc. of blood.

50 units of insulin were given subcutaneously, the stomach washed out, an enema administered, a subpectoral injection of 1800 cc. of 2.5 per cent glucose was given, followed by 800 cc. of glucose solution intravenously. Insulin at the rate of 50 units per hour together with the application of heat and the other measures resulted in the return of a conscious state in about $3\frac{1}{2}$ hours.   During the next few days he had several severe insulin reactions and throughout the hospital stay it was not found possible to keep the urine consistently sugar-free.   He was discharged from the hospital on November 13, 1928, with a diet of carbohydrate 95, protein 80, and fat 135, and insulin to be taken 20 units before breakfast and 10 units before supper.

THE RATE OF HYDROLYSIS OF RIBONUCLEOTIDES.

BY P. A. LEVENE AND ERIK JORPES.[*]

(From the Laboratories of The Rockefeller Institute for Medical Research, New York.)

The knowledge of the structure of a nucleotide may be considered complete when, in addition to the knowledge of the structure of the base and of the sugar, there is available also the information of the allocation of the sugar on the base and of the phosphoric acid on the sugar. Such information has been furnished in the case of inosinic acid and our knowledge of the structure of this nucleotide may be regarded as complete. Our knowledge of the structure of the nucleotides which compose the molecule of the complex nucleic acids is less perfect. Nucleotides of two types enter into the structure of these acids, those in which the base is a purine derivative and those in which it is a pyrimidine derivative.

Direct evidence as to the allocation of the phosphoric acid radical in these nucleotides is still lacking. It was known, however, that the stabilities of the phosphoric acid radicals differed in the nucleotides of these two types. The rates of hydrolysis of the phosphoric acid of the pyrimidine nucleotides were found by Yamagawa,[1] working in this laboratory, to be of the same order of magnitude as the rate of inosinic acid. The rates of hydrolysis of the phosphoric acid of the purine nucleotides were of a higher order of magnitude. It therefore seemed warranted to conclude that in the pyrimidine nucleotides, as in inosinic acid, the phosphoric acid is attached to the end carbon atom. However, it was always realized that the conclusion needed to be tested by a more direct method.

Recently methods have been developed in this laboratory which permit the establishment, without much difficulty, of the

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[1] Yamagawa, M., *J. Biol. Chem.*, **43**, 339 (1920).

TABLE I.

Influence of Acid Hydrolysis On Some Purine and Pyrimidine Ribose Nucleotides.

Sample.	Strength of acid. N	10 min. P in free phosphates Found mg. P per cc.	10 min. P in free phosphates In per cent of total.	10 min. Reducing power Found mg. ribose per cc.	10 min. Reducing power In per cent of calculated amount.	30 min. P in free phosphates Found mg. P per cc.	30 min. P in free phosphates In per cent of total.	30 min. Reducing power Found mg. ribose per cc.	30 min. Reducing power In per cent of calculated amount.	60 min. (N acid) or 120 min (0.1 N) P in free phosphates Found mg. P per cc.	60 min. P in free phosphates In per cent of total.	60 min. Reducing power Found mg. ribose per cc.	60 min. Reducing power In per cent of calculated amount.
I. Adenylic acid from yeast. 1 cc. contained 0.852 mg. P corresponding to 4.12 mg. ribose.	1	0 62	72 8	3 31	80 4	0 62	72 8	3 44	83 5	0 75	88 0	3 72	90 4
	0 1	0 094	11 0	0 53	12 9	0 34	39 9	1 60	38 8	0 60	70 4	3 39	82 3
II. Guanylic acid from pancreas. 1 cc. contained 0.702 mg. P corresponding to 3.40 mg. ribose.	1	0 525	74 8	2 88	84 5	0 63	89 6	3 15	92 6	0 64	91 2	3 01	88 5
	0 1	0 060 ?	8 6 (?)	0 54	15 9	0 185	26 4	1 32	38 8	0 50	71 3	2 63	77 3

III. Hydrogenated cytidine phosphoric acid from yeast.													
Experiment 1. 1 cc. contained 0.867 mg. P corresponding to 4.19 mg. ribose.	2	0 586	67 5	2 99	71 3	0 65	75 0	3 22	76	80 71	82 0	3 04	72 5
	0 1	0 089	10 3	0 52	12 4	0 159(?)	18 4(?)	1 18	28	20 45	52 0	2 35	56 0
Experiment 2. Same solution.	1					0 595	68 7	3 0	71 5	0 50	57 6	2 01	48 0
	0 1												
IV. Cytidine phosphoric acid from yeast. 1 cc. contained 0.942 mg. P corresponding to 4.56 mg. ribose.	1	0 100	10 6	0 45	9 9	0 109	11 6	0 43	9 40	146	15 5	0 60	13 3
	0 1	0 057(?)	6 1(?)	0 12	2 6	0 060	6 4	0 34	7 50	130	13 8	0.47	10 3
V. Inosinic acid. Experiment 1. 1 cc. contained 0.654 mg. P corresponding to 3.17 mg. ribose.	1	0 027	4 13	3 02	95 1	0 084	12 9	3 03	95 50	116	17 7	2 82	89 0
	0 1	0 010	1 53(?)			0 017	2 6	2 96	93 30	069	10 5	3 06	96 5
Experiment 2. 1 cc. contained 1.160 mg. P corresponding to 5.61 mg. ribose.	1	0 070	6 03	1 92	34 2	0 131	11 3	5 18	92 3	0 172	14 8	5 30	94 5
	0 1												

allocation of a substituting group on a sugar.[2] The time therefore seemed opportune to resume the work on the allocation of the phosphoric acid in both the purine and pyrimidine nucleotides. The methods developed for this purpose require the preparation of the phosphoric ester of the sugar; in the case of ribonucleotides, this compound is the phosphoric ester of ribose   It may be mentioned that efforts to methylate either nucleotides or nucleosides proved unsuccessful. The preparation of the phosphoric esters from the nucleotides is possible only in the case when the rate of hydrolysis of the base is higher than that of the phosphoric acid radical. This condition was satisfied in the case of inosinic acid. We have now undertaken to determine the respective rates of hydrolysis of the adenosine nucleotide (adenylic acid) prepared from yeast nucleic acid. The result is given in Table I.

From Table I it is seen that in adenylic acid the hydrolysis of the base and the hydrolysis of the phosphoric acid proceed approximately at the same rate, in contrast to the behavior of inosinic acid. Under such conditions the hope of ascertaining by the existing methods the allocation of the phosphoric acid in adenylic acid had to be abandoned. No such difficulty was anticipated in the case of the pyrimidine nucleotides in the light of previous experience on stability of the phosphoric acid radical in these nucleotides. In the pyrimidine nucleotides, however, not only the phosphoric acid, but also the base, is hydrolyzed only with great difficulty. Levene and La Forge, however, have found that the rate of hydrolysis of the base from the pyrimidine nucleosides can be enhanced by hydrogenation of the base. Therefore the pyrimidine nucleotides were hydrogenated prior to subjecting them to hydrolysis. The progress of hydrolysis of the hydrogenated and non-hydrogenated pyrimidine nucleotides is given in Table I. It was surprising to find that the hydrogenated cytidine phosphoric acid behaved exactly like the purine nucleotides, not only with respect to the rate of hydrolysis of the base, but also with respect to that of the phosphoric acid. *Hence the earlier conclusion*

[2] Levene, P. A., and Sobotka, H., *J. Biol. Chem.*, **71**, 471 (1926–27). Levene, P. A., and Wintersteiner, O., *J. Biol. Chem.*, **75**, 315 (1927). Levene, P. A., and Wolfrom, M. L., *J. Biol. Chem.*, **77**, 671, (1928). Levene, P. A., and Raymond, A. L., *J. Biol. Chem.*, **80**, 633 (1928). Levene, P. A., and Mori, T., *J. Biol. Chem.*, **81**, 215 (1929).

as to the allocation of the phosphoric acid in the pyrimidine nucleotides has to be abandoned in favor of the view that the phosphoric acid in these nucleotides as well as in the purine nucleotides is allocated on a secondary carbon atom.   The question naturally arose whether all the ribomononucleotides occurring in animal tissues as such and not as parts of nucleic acid resemble inosinic acid in the allocation of the phosphoric acid.   From Table I it is seen that the guanylic acid derived from the pancreas gland behaves exactly like the adenylic acid derived from yeast nucleic acid. *Thus it is evident that not all ribomononucleotides occurring in animal tissues are derived from an identical phosphoribose.*

EXPERIMENTAL.

The samples of adenylic and cytidine phosphoric acids used were prepared from yeast nucleic acid in earlier work in this laboratory and analyzed as pure.   The free cytidine phosphoric acid was hydrogenated in aqueous solution with platinum oxide (prepared according to the instructions given by Adams and Shriner[3]) as catalyst.   An attempt was made to use palladium oxide, but without success.   The hydrogenation of the pyrimidine base, however, proceeded very slowly.   Although the reduction in Paal's apparatus was continued 36 hours, it seemed not to be complete, both the reducing power and the amount of free phosphates after hydrolysis being less than in the corresponding experiments with purine nucleotides.

The guanylic acid was prepared from pancreas according to the procedure described by Jones and Perkins.[4]   A further purification through the silver and brucine salts was performed.   The free guanylic acid analyzed air-dried as follows:

53.60 mg. substance: 6.85 cc. 0.1 N HCl.
 4.39 "   "   : 23.65 mg. ammonium phosphomolybdate.
     $C_{10}H_{14}N_5PO_8 \cdot 2H_2O$.   Calculated.   N 17.54, P 7.77.
                                Found.   " 17.90. " 7.80.

The inosinic acid was prepared in this laboratory from fish extract by Dr. T. Mori as the crystalline barium salt.   Dried at 100° under reduced pressure it analyzed as follows:

[3] Adams, R., and Shriner, R. L., *J. Am. Chem. Soc.*, **45**, 2171 (1927).
[4] Jones, W., and Perkins, M. E., *J. Biol. Chem.*, **62**, 291 (1924–25).

50.00 mg. substance: 4.09 cc. 0.1 N HCl.
50.00 "   "   : 22.6 mg. BaSO$_4$.
 4.30 "   "   : 19.745 mg. ammonium phosphomolybdate.
    C$_{10}$H$_{11}$O$_8$N$_4$PBa.   Calculated. N 11.59, P 6 40, Ba 28.41.
                Found.   " 11.46, " 6.66, " 26.60.

From the nucleotides stock solutions were prepared. These were then diluted with hydrochloric acid. The final strength of the acidity and the concentrations of the nucleotides are given in Table I. When alkali was employed to dissolve the nucleotides, a corresponding amount of acid was added in excess.

The hydrolysis was performed in sealed tubes at the temperature of a boiling water bath. The usual amount of solution in each tube was 3 cc. After cooling, the solutions were neutralized with sodium hydroxide and diluted to 5 cc. each. In the samples containing guanylic acid guanine settled out on neutralization and was removed through centrifugalization.

On these solutions the reducing power and the amount of phosphoric acid set free during hydrolysis were determined, the former according to the method of Hagedorn and Jensen[5] and the latter either colorimetrically according to Kuttner and Cohen[6] or gravimetrically according to Pregl-Lieb after previous precipitation with magnesia mixture. The sugar tests were made in duplicate with the usual close agreement which this method gives. As the reducing power of a sample of pure dry d-ribose, taken from a collection of carbohydrates in this laboratory, was found to be only 87.3 per cent of that of glucose, the figures taken from the Hagedorn-Jensen table were correspondingly increased. The amount of sugar found by titration is hence expressed as ribose.

Gravimetric phosphorus determinations after previous precipitation with magnesia mixture were made in the experiments on guanylic acid and in Experiment 2 on inosinic acid. They are to be considered as rather accurate, whereas the colorimetric determinations in the experiments with the smallest quantities of free phosphoric acid deviated considerably in duplicate. They express, however, in every case the right order of magnitude of the amount of phosphoric acid split off by hydrolysis.

[5] Hagedorn, H. C., and Jensen, B. N., Z. physiol. Chem., **137**, 92 (1923).
[6] Kuttner, T., and Cohen, H., J. Biol. Chem., **75**, 577 (1927).

A DESCRIPTION OF THE GLASS ELECTRODE AND ITS USE IN MEASURING HYDROGEN ION CONCENTRATION.

By A. E. MIRSKY and M. L. ANSON.

(From the Hospital of The Rockefeller Institute for Medical Research, New York, and the Laboratories of The Rockefeller Institute for Medical Research, Princeton.)

This paper contains the description of a glass electrode and of the apparatus needed for its use. If a glass membrane is interposed between two solutions of different hydrogen ion concentration, a potential difference will be set up across the membrane. Under certain conditions this P.D. is determined by the difference in pH of the two solutions so that if the pH of one is known, that of the other can be calculated. This method of measuring pH is especially well adapted to many biological purposes. The glass electrode is as accurate as the hydrogen electrode (within the pH range of biological significance), as rapidly operated as the quinhydrone electrode, and more widely applicable than either of the other two. It is unnecessary here to discuss the theory of the potentials obtained. Nevertheless some mention of the development of the glass electrode may be of value. Cremer (1) while making a study of bioelectric phenomena was the first to observe that the magnitude of the potential at a glass membrane is affected by the difference in acidity of the two solutions on either side of the membrane. Exact measurements of this effect were made by Haber and Klemensiewicz (2) who found that the following equation expresses the relation between the hydrogen ion concentrations of the solutions and the potential at the glass membrane

$$E = RT \ln \frac{C_{H_1}}{C_{H_2}}$$

The membrane potential may be regarded as consisting of two glass-water interfacial potentials.   Under these circumstances this equation can then be derived if it is assumed that the hydrogen ion concentration of the glass phase remains constant.   Recently Hughes (3) has pointed out that the hydrogen ion concentration in the glass phase may be held relatively constant by the buffer action of the glass which is a mixture of the salt of a weak acid (Na_2SiO_3) with the anhydride of that acid (excess SiO_2). At about pH 9 the divergence in values of pH between the glass and hydrogen electrodes becomes noticeable and it is here that the buffer action of the glass would be less effective since the apparent first dissociation constant of H_2SiO_3 is about 10^{-9}. The apparent second dissociation constant of H_2SiO_3 is 10^{-13} and it is at pH 13 that the divergence between the glass and hydrogen electrodes rapidly increases.   Haber suggested that the glass cell could be used to measure hydrogen ion concentration.   There the matter rested, however, until more than 15 years later Kerridge (4) made exact pH measurements with it.   She showed that under certain conditions only the difference in hydrogen ion concentration affects the P.D. and that the measurements made with the glass electrode agree to within 0.01 pH with those made with the hydrogen electrode.   The practical applications of the glass electrode begin with her contribution.   The arrangement which we employ is derived directly from hers.   We have made a number of changes, however, that make the construction and use of the electrode simpler.

1. The Electrode.—The electrode is made of ordinary soft soda glass.   The glass we used was supplied by the Kimball Glass Company of Vineland, New Jersey.   It has the following analysis:

	per cent
Silica	69.7
Alumina, iron oxide, *etc*.	2.0
Zinc oxide (with small amount of manganous oxide).........	1.5
Calcium oxide	6.0
Magnesia..	3.8
Boric anhydride.....	0.8
Sodium oxide (including small amount of potassium oxide)..	15.7

A borosilicate glass, such as Pyrex, cannot be used.   Hughes (3) has recently tested a variety of soda glasses and finds that those of low alumina content are most suitable.

To make the electrodes, glass tubing having walls of medium thickness and an inside diameter of $\frac{3}{8}$ inch is used. A small bulb is blown at one end with walls thick enough to be strong. By directing a fine small flame at a point on the bulb and then sucking at the open end of the tube a small cup is formed depressed below the surface of the bulb. The cup should contain about 0.5 to 1.0 cc. and should be made quickly and without annealing (3). The total length of bulb and tube is about 3 inches. The
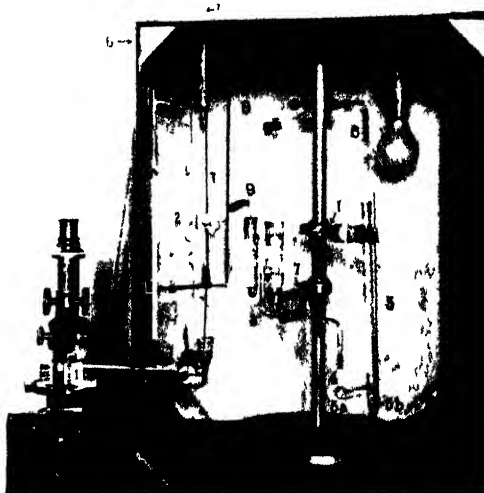
Fig. 1 1, Lindemann electrometer; 2, switch; 3, calomel half-cell; 4, agar tip; 5, combined glass electrode and calomel half-cell; a, glass electrode; b, calomel half-cell; 6, metallic shielding; 7, quartz insulation; 8, wire to potentiometer; 9, wire to electrometer needle.

open end of the tube is joined at right angles to a longer piece of the same kind of tubing (Fig. 1). The longer tube has a length of about 10 inches. It is closed at one end and the junction with the shorter tube is made about 1 inch from the closed end. If the vertical arm of the electrode were not so long, it would be necessary before making a measurement to dry carefully the horizontal arm so as to prevent electrical leakage. The electrode is cleaned with chromic acid, carefully rinsed with tap water, and allowed to stand in water for 2 days, or more if necessary.

The thin wall of the cup is the glass membrane. A buffer solution is placed in the large bulb, and the solution of unknown pH in the small one. The P.D. across the membrane can then be measured. Electric contact with each solution is made by means of a calomel half-cell. The system has been simplified by placing one of the calomel half-cells instead of a buffer solution inside the large bulb. The arrangement of the combined glass electrode and half-cell is shown in Fig. 1.

2. Calomel Half-Cell.—A convenient form of calomel half-cell is shown in Fig. 1. It is supported in a collar so that it can be easily moved from side to side. It can also be moved vertically by a rack and pinion. When the tip of the saturated KCl bridge dips into the cup of the glass electrode, the KCl, being heavier than water, flows down to the glass membrane where it constantly keeps changing the existing P.D. and prevents reproducible measurements from being made. This difficulty can be prevented by joining an agar-KCl tip to the end of the bridge.

3. Measurement of Potential.—The resistance of the glass membrane is so high that a galvanometer cannot be used in connection with a potentiometer to measure the P.D. across the membrane. An electrometer must therefore be used instead. The Lindemann electrometer (5) as pointed out by Kerridge, is a convenient form of this instrument, requiring no levelling and being nearly as sensitive as other electrometers. The use of the Lindemann electrometer is fully described in Kerridge's paper (4) where a description of the wiring is also to be found. The electrometer needle is earthed except when connected to the glass electrode. A convenient method for switching the needle from ground to the electrode has been devised (Fig. 2). The switch should be constructed so that when it is used the electrometer needle does not move at all if the P.D. of the glass cell is exactly balanced by the P.D. of the potentiometer system. With an ordinary switch the mere act of switching may cause the needle to jump (and then return to its zero) even at the null-point. The wire from the electrometer needle divides at 4, one part going to 5 and thence to earth, while the other goes to 7 and thence to the glass electrode. When the rod, 2, is pressed down the contact at 5 is broken and that at 7 is made, the latter being made the *instant* after (or even before) the former is broken. When the rod is released the re-

verse contacts are made. This switch facilitates the use of the electrometer as a null-point instrument. The apparatus is shielded from electrostatic disturbances by the metal box, 9, and
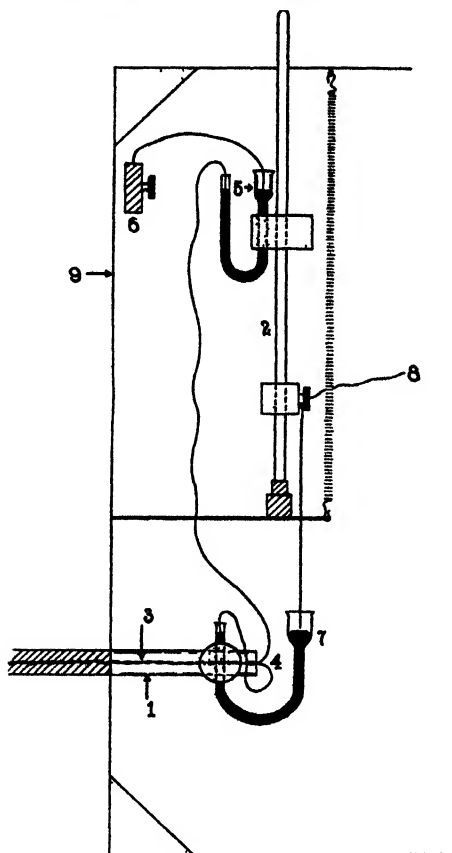
Fig. 2. The switch. 1, quartz tube; 2, quartz rod; 3, wire to electrometer; 4, wire to electrometer divides; 5, contact for earthing; 6, to earth; 7, contact to electrode; 8, to glass electrode; 9, shielding.

tube which are earthed. It has not been necessary to close the open side of the box.

4. *Insulation.*—As a consequence of the great resistance inter-

posed in the circuit by the glass membrane that part of the circuit
between the electrode and electrometer must be carefully insu-
lated. Kerridge used amber for this purpose, but we find that it
is accomplished just as satisfactorily and more conveniently with
quartz. Insulation is necessary in each of the arms holding the
electrode and calomel half-cell where there is a short length of
quartz rod. For the same reason the switch leading from elec-
trode to electrometer has certain parts made of quartz, as indi-
cated in Fig. 2.

5. *Manipulation.*—When not in use the glass electrode is kept
emersed in water. The water is withdrawn from the small cup
by means of a small pipette furnished with a rubber nipple at
one end; to the other end a piece of flexible capillary rubber
tubing about $1\frac{1}{2}$ inches long is attached to avoid breaking the
glass membrane. To clean the electrode after a measurement
has been made, all that it is usually necessary to do is to rinse
the small bulb and agar tip with a stream of distilled water. It
takes about 1 minute to make a measurement. The P.D. of the
glass electrode is measured first when it contains a solution of
known pH (*e.g.* 0.05 M potassium hydrogen phthalate). The pH
is observed again when the solution of unknown pH is added to
the cup of the electrode. From the two, the unknown pH can
be calculated. Kerridge has shown that

$$pH_x = 3.97 + \frac{E_s - E_x}{0.000,198,37 \; T}$$

E_s is the potential found when potassium hydrogen phthalate is
in the electrode, E_x the potential when the unknown solution
has been added, and T is the absolute temperature. Any po-
tential due to the glass itself does not affect the final result.

It may be well to summarize the advantages of the changes
which have been made from Kerridge's arrangement.

Electrode.—The present electrode is easier to make than the
ones described by Kerridge; it has a greater insulation surface,
and it contains in it one of the necessary calomel half-cells.

Insulation.—Quartz is stronger and easier to clean than amber.

Switch.—A switch has been described which combines speed of
change of contact with ample insulation.

KCl Contact.—The contact is made with an agar tip instead of through a ground glass joint, which is less convenient and takes more room.

SUMMARY.

The construction and use of a glass electrode for the measurement of hydrogen ion concentration is described.

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THE HEMOGLOBIN CONCENTRATION OF THE BLOOD
OF MARINE FISHES.

BY F. G. HALL AND I. E. GRAY.

*(From the Zoological Laboratory of Duke University, Durham, North Carolina,
and the Zoological Laboratory of Tulane University, New Orleans.)*

It has been said that hemoglobin is the most interesting substance in the world. Though this may not be the opinion of everyone, it assuredly seems true that with each succeeding year new properties are assigned to hemoglobin which give it a more and more prominent place in physiology. Much credit is certainly due to Barcroft (1928) for designation of many of the properties of hemoglobin and for recognition of the important rôle that it plays in nature.

Hemoglobin is widely distributed among animals. It occurs in the blood of several invertebrates and is present in the blood of all vertebrates. Its concentration varies greatly in different animals that have been studied. Likewise its composition appears to vary with each species. Generally hemoglobin is a compound of globin with hematin. A molecule of hematin contains 1 atom of iron. This atom of iron enables hemoglobin to carry oxygen in large amounts. Therein lies the chief, although not the sole, function of hemoglobin in organisms.

In a study of the influence of asphyxia upon fishes (Hall, Gray, and Lepkovsky, 1926) it was observed that individual fishes showed a marked increase in hemoglobin concentration during asphyxia. It was also incidentally observed that several different species showed a considerable variation in the normal concentration of hemoglobin although there was little shown among individuals of the same species. This suggested that a comparative study of the hemoglobin concentration of fishes inhabiting similar waters would be important. Such analyses were carried on in the laboratory of the United States Bureau of Fisheries at Woods Hole, Massachusetts. Fishes representing fifteen

common species of teleosts and two elasmobranchs of the Woods Hole region were caught in traps and carefully removed to live cars where they were allowed to swim freely, without danger of asphyxia, for a day. The results obtained from elasmobranchs are not to be compared directly with those obtained with teleosts, but otherwise it is believed that the results are comparable throughout.

Methods.

The hemoglobin concentration was determined by quantitative analyses of samples of blood for iron. Results of preliminary analyses for oxygen-carrying capacity, amount of hematin, carbon monoxide capacity, and iron indicated that, for a comparative study of the hemoglobin concentration in fishes, the iron method would yield the most satisfactory results. Wong's (1928) method was adopted for this purpose and the following procedure employed.

Fishes were bled from the caudal artery by severing the tail. The blood was collected into an Erlenmeyer flask containing sufficient lithium oxalate to prevent clotting. 1 cc. of oxalated blood was pipetted into a 50 cc. volumetric flask to which had been added 2 cc. of iron-free, concentrated sulfuric acid. The flask was agitated as the blood mixed with the acid. 2 cc. of saturated potassium persulfate were added not less than 10 minutes later. Dilution with 20 cc. of distilled water was then made, after which 2 cc. of 10 per cent sodium tungstate solution were added. The volumetric flask was now shaken and cooled to room temperature. The solution was diluted to 50 cc. volume and filtered through a dry filter paper into a dry test-tube. 20 cc. of the filtrate were pipetted into a graduated test-tube. Into a similar test-tube 1 cc. of a standard ferrous ammonium sulfate, containing 0.1 mg. of Fe per cc., was pipetted. 0.8 cc. of concentrated sulfuric acid was added to the standard and dilution to 20 cc. made. The solution was cooled to room temperature.

To both the standard and the blood solution 1 cc. of saturated potassium persulfate was added. 4 cc. of a 3 N solution of potassium sulfocyanate, which contained 40 cc. of acetone per liter, were pipetted. After mixing by several inversions of the test-tubes the unknown solutions were read at once against the standard on a

TABLE I.

Species of Fish Experimented upon.

Common name.	Scientific name.	Family.	Order.
Teleosts.			
Eel.	*Anguilla rostrata* (LeSueur).	Anguillidæ.	Apodes.
Menhaden.	*Brevoortia tyrannus* (Latrobe).	Clupeidæ.	Isospondyli.
Mackerel.	*Scomber scombrus* Linnæus.	Scombridæ.	Percomorphi.
Bulls-eye mackerel.	*Pneumatophorus colias* (Gmelin).	"	"
Bonito.	*Sarda sarda* (Bloch).	"	"
Butter-fish.	*Poronotus triacanthus* (Peck).	"	"
Scup.	*Stenotomus chrysops* (Linnæus).	Sparidæ.	"
Rudder-fish.	*Palinurichthys perciformis* (Mitchill).	Centrolophidæ.	"
Cunner.	*Tautogolabrus adspersus* (Walbaum).	Labridæ.	Pharyngognathi.
Puffer.	*Spheroides maculatus* (Bloch and Schneider).	Tetraodontidæ.	Plectognathi.
Sea-robin.	*Prionotus carolinus* (Linnæus).	Triglidæ.	Cataphracti.
Silver hake.	*Merluccius bilinearis* (Mitchill).	Merlucciidæ.	Anacanthini.
Toadfish.	*Opsanus tau* (Linnæus).	Batrachoididæ.	Jugulares.
Goosefish.	*Lophius piscatorius* Linnæus.	Lophiidæ.	Pediculati.
Sand-dab.	*Lophopsetta maculata* (Mitchill).	Pleuronectidæ.	Heterosomata.
Elasmobranchs.			
Electric ray.	*Narcacion nobilianus* Bonaparte.		
Smooth dogfish.	*Mustelus canis* (Mitchill).		

Bausch and Lomb biological colorimeter. With samples of
blood containing small amounts of iron a standard of 0.05 mg.
of Fe per cc. was used. The mg. of Fe per 100 cc. of blood were
then calculated. Since the absolute amount of iron contained
by the hemoglobin of fishes is not known (it probably varies but
little from that of mammalian hemoglobin), it was thought to be

TABLE II.

Comparison of Iron Concentration in Blood of Marine Fishes.

Species.	No. of individuals.	Iron per 100 cc. of blood.		
		Low.	High.	Average.
		mg.	*mg.*	*mg.*
Teleosts.				
Bonito...............................	3	37.0	52.9	45.5
Bulls-eye mackerel......................	9	37.4	48.7	43.8
Common "	16	34.3	51.0	43.0
Menhaden............................	18	25.1	45.0	41.0
Cunner.............................	4	24.8	31.9	27.7
Butter-fish...........................	8	21.8	38.5	27.4
Scup................................	11	20.3	35.6	25.3
Sea-robin............................	8	21.7	27.6	23.7
Rudder-fish..........................	8	17.3	25.0	21.7
Puffer...............................	8	17.1	27.8	21.5
Eel.................................	5	16.9	22.0	20.4
Silver hake..........................	8	16.3	24.4	19.4
Goosefish............................	4	11.1	16.6	14.7
Toadfish.............................	3	12.5	15.2	13.5
Sand-dab............................	5	7.7	13.1	11.5
Elasmobranchs.				
Smooth dogfish.......................	5	11.1	17.4	15.4
Electric ray..........................	4	6.7	10.0	8.8

more accurate to leave the results expressed in concentration
of iron.

Results.

Results were obtained from fifteen species of teleosts which
represent ten orders and thirteen families and from two species of
elasmobranchs as shown in Table I.

Results of the analyses of the blood of fishes are summarized
in Table II. The species are arranged in order of their iron con-

centration with the bonito, which has the highest value, first, and the sand-dab, which, except for the elasmobranchs, is lowest, last. The iron concentration of the blood of teleosts was found to vary from 11.5 mg. to 45.5 mg. per 100 cc. Thus it appears that the bonito had 4 times as much iron per unit volume of blood as does the sand-dab. One will also observe that all representatives of the mackerel family, Scombridæ, showed similar iron values. Both species of elasmobranchs showed small concentrations of iron when compared with the majority of teleosts. The limits of individual variations are shown by giving the high and low values. Most of the analyses gave results which fluctuated quite close to the average figure.

DISCUSSION.

Apparently there is a general correlation between the habits of fishes and the hemoglobin concentration of their blood. This is illustrated by the fact that the first four species in Table II, which are those having the highest iron values, are all pelagic forms with similar habits, although four genera and two families are represented. They are known to fishermen as the most active and acquire the greatest speed in their movements from place to place. It is difficult to keep them alive when confined in aquaria because of their great activity.

The last three fishes, namely goosefish, toadfish, and sand-dab, are inactive and sluggish. They may rest on the bottom for long periods without any sign of life. This is an extreme contrast to mackerel which are constantly moving rapidly through the water. The goosefish is reported to angle for its food, using a fringe on its first dorsal spine as a lure. It has an insatiable appetite, eating any animal which may be up to one-half the mass of its own body. Its meals are, however, quite irregular as to time. They depend little upon the activity of the goosefish, but more upon animals that come by chance within its reach and are attracted by its bait. Here again is a marked contrast to the mackerel, which depends upon a constant source of food—plankton— which is obtained by great activity and constant effort. The toadfish is very similar to the goosefish in its habits. Other species are intermediate between these two extremes; some are

nearer to the active forms, illustrated by scup, butter-fish, and cunner, while others such as the hake and eel resemble the inactive group.

In a previous paper (Hall, 1929) it has been shown that scup have a relatively high oxygen consumption when at rest. This is in sharp contrast to the toadfish which has a very low rate of metabolism. Menhaden have been found to have a high oxygen consumption. Scup have also been shown to have their oxygen consumption influenced but little by variations in oxygen tension between 30 to 120 mm. of Hg, while on the other hand the oxygen consumption of the toadfish was found to be directly proportional to variations in the oxygen tension. The activity of the mackerel is so great that it has not been possible to obtain any idea of the basal or standard metabolism.

It is also an interesting point for speculation that the four species having the highest hemoglobin concentration are migratory fishes, while those having low concentrations are largely nonmigratory. Perhaps available hemoglobin limits the extent to which fishes may migrate through the ocean.

It is indicated, therefore, that the hemoglobin concentration of marine fishes is perhaps an index of their physiological activity. We may have in the fishes an illustration of how hemoglobin because of its function in respiration permits organisms to attain habits of definite survival value.

SUMMARY.

1. The normal hemoglobin concentration of the blood of seventeen species of marine fishes was determined.

2. The more active species appear to have the highest hemoglobin concentration and the less active species have the lowest concentration.

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THE FLUCTUATIONS OF THE CAPILLARY BLOOD SUGAR IN NORMAL YOUNG MEN DURING A TWENTY-FOUR HOUR PERIOD (INCLUDING A DISCUSSION OF THE EFFECT OF SLEEP AND OF MILD EXERCISE).*

By HARRY C. TRIMBLE AND STEPHEN J. MADDOCK.†

(*From the Biochemical Laboratory and the Laboratory for Surgical Research of Harvard Medical School, Boston*)

The response of the human organism to the ingestion of a single dose of carbohydrate, as determined by the sugar content (total reducing substance) of the blood, has been extensively studied in both normal and diabetic subjects   Yet when search of the literature is made for the fluctuations of the blood sugar over periods much longer than 2 hours after carbohydrate intake or for the effect of repeated meals of mixed composition, a surprising paucity of data is found.   This is even more true when the capillary blood is under consideration.   Our interest in these matters arose in connection with some studies upon long continued hypoglycemia (1).   In that work the variations of the blood sugar in diabetic patients subsisting upon their regular diets were followed for a 24 hour period.   When it became desirable for the purpose of comparison to have similar data upon normal individuals living in their customary manner, we could find no papers which gave blood sugar values for an entire day.   Also some random isolated observations suggested to us that the sugar content of capillary blood might be found at elevated levels at periods considerably later with reference to food ingestion than tolerance tests based upon analysis of venous blood indicate.   Therefore, in several experiments we have determined the sugar content of

the capillary blood of a group of normal young men at hourly intervals for a complete day under conditions as normal as possible. The results, while not altogether novel, contain some features of general interest. Jonas, Miller, and Teller (2) have determined the concentrations of sugar in the blood of six non-diabetic convalescent patients over a period of 12 hours. Venous blood only was analyzed and the conclusion was drawn that the blood sugar concentration falls back to the fasting level in 1 to 2 hours after a meal. In three of the cases the maximum rise occurred after breakfast but since blood samples were taken at hourly intervals the highest point might well have been missed in some of the experiments. Leire (3) and Jacobsen (4) have reported a few brief experiments upon non-diabetic hospital patients. Petrén (5) and Brill (6) have studied normal subjects. Petrén gave to eight persons a mixed meal containing an average of 77 gm. of carbohydrate. At the end of the 1st hour the maximum sugar concentrations were observed, while the return to the fasting level did not occur until between the 3rd and 4th hour. Bang's (7) method of analysis was employed and all experiments were confined to the morning hours. Brill performed experiments upon thirteen normal subjects at breakfast time. Two blood samples per person were taken, one before, and the other 1½ hours after ingestion of a mixed meal containing 100 gm. of carbohydrate, 26 gm. of protein, and 27 gm. of fat. In three cases the sugar content of the second sample increased slightly above the fasting level, 12 mg. being the maximum. In seven cases the second sample showed a decrease, −19 mg. being the greatest. In three cases there was no change. The experimental methods employed are not stated. Friedenson, Rosenbaum, Thalheimer, and Peters (8) have just reported some studies in which the concentrations of sugar in the capillary and venous bloods were compared after the ingestion of either 50 gm. of glucose or a mixed breakfast of 70 or more gm. carbohydrate content. A total of twenty-four experiments upon twenty normal individuals is recorded. Blood samples were taken at 30, 60, 90, and 120 minutes after ingestion of the carbohydrate. For the analytical procedure a micro modification of Benedict's (9) 1925 blood sugar method was employed. At an interval of 30 minutes after ingestion of food these investigators found that a definite arterial

hyperglycemia was established which usually still existed at the end of 1 hour.   It was usually higher at 30 rather than at 60 minutes.   In two-thirds of the experiments the capillary blood sugar had returned to the normal fasting range at the end of 90 minutes. These observers concluded that the characteristics of the normal arterial (cutaneous) and venous blood sugar after 50 gm. of glucose or a mixed breakfast are indistinguishable.   Reference to the work of some other investigators which has a bearing upon particular points in connection with our findings will be made at appropriate places in the sections which follow

EXPERIMENTAL.

Methods.

The subjects of the experiments to be described were medical students all of whom resided in and took their meals in the dormitory just across the street from this laboratory.   Thus they were readily accessible at all times while they carried on their usual activities.   The experiments were started in the morning just before the breakfast hour when the first sample of blood was drawn.   From that time on other samples were obtained every hour for the ensuing 24.   During the day the subjects came into our laboratory to permit us to withdraw the blood, while during the evening and night one of us called at the rooms and collected the samples while the students were at work or asleep.   In many instances sleep was not interrupted by the procedure necessary to obtain the blood.

Unless otherwise indicated samples of blood were drawn from the fingers into a Folin micro pipette and then immediately discharged into a solution of tungstic acid for precipitation of protein. The solutions of tungstic acid used were less than 24 hours old and did not contain any preservative.   In order to insure uniformity all of the blood samples were collected and measured by one operator.   A quick thrust by a sharp 18 gage antitoxin needle usually produced the required 0.1 cc. of blood to fill the pipette. Sometimes gentle milking of the finger was necessary when the blood flowed slowly.   For determination of the sugar content of the blood samples the directions of Folin (10) were followed.   The colorimetric measurements were made by one individual as soon

as possible after the blood was collected and the identity of the samples usually was not known until the observation had been completed.

In some of the preliminary experiments when comparison measurements upon the sugar content of capillary and of venous blood was desired, samples were obtained simultaneously from a finger puncture and from a hollow needle inserted into the median cubital vein. The first portion of the venous blood was collected in a syringe, which was then detached and the blood allowed to flow directly from the needle into a Folin micro pipette. The contents of the syringe were immediately treated by the Folin-Wu (11, 12) procedure and the contents of the micro pipette by the Folin (10) micro technique.

Glucose from the Bureau of Standards was used for the preparation of all of the colorimetric standards.

The food consumed during the experiments was that contained in ordinary mixed meals. As regards the carbohydrate-rich items the intake for the different subjects was largely the same and institutional control insured a considerable degree of uniformity in the quantities served. The average weights of a number of representative helpings of the different articles involved was determined and then the approximate carbohydrate content was computed by the aid of standard clinical tables. We are well aware that this procedure did not yield precise results regarding the carbohydrate intake. Absolute information upon this point could be obtained only by the laborious undertaking of actually analyzing everything as served, or by restriction of the diet to a pure carbohydrate. If this last procedure had been employed for a series of meals it would have represented an unphysiological condition and moreover the information gained would not be expected to differ greatly from that already available in the records of glucose tolerance tests. Our primary interest was in obtaining a general survey of blood sugar fluctuations throughout a day under conditions as normal as possible and we were not attempting to account quantitatively for the food ingested. Therefore, we decided not to follow the more exact procedures for estimation of the carbohydrate consumed. Consequently, in interpreting our data where the effect of meals is concerned we have not discussed individual differences but have paid attention only to the group

averages, whereby the effect of the individual variations of intake is largely eliminated.

Preliminary Experiments.

Most of the data which have been recorded regarding fluctuations of blood sugar have been obtained with venous blood and frequently the method of measurement has been by the procedure of Folin and Wu.　Since the experiments which we are reporting

TABLE I.

Simultaneous Sugar Content of Capillary Blood (Folin Ferricyanide Method) and of Venous Blood (Folin-Wu Method and Folin Ferricyanide Method).

Subject No.	Finger blood. Folin ferricyanide method. (1)	Venous blood.		
		Folin-Wu method (2)	Folin ferricyanide method.	
			On diluted Folin-Wu filtrate.* (3)	On directly collected blood.† (4)
	mg. per cent	*mₒ per cent*	*mg. per cent*	*mg. per cent*
I	99	99	95	98
II	96	100	97	97
III	98	101	96	95
IV	91	95	91	93
V	91	92	89	90
VI	89	92	89	89
Average.	94 0	96 5	93 3	93 6

* The blood filtrate prepared for the Folin-Wu method (Column 2) was diluted 10 times for these measurements.

† Blood for the determinations recorded in Column 4 was allowed to flow into a micro pipette directly from the same hollow needle which had been used to obtain the sample for the Folin-Wu method (Column 2).

deal with capillary blood and since a new method of analysis is employed, it was necessary to study the effect of the changed conditions in order to interpret our data in terms of older standards.　In Table I we present a comparison of the apparent blood sugar (total reducing substances) of fasting individuals when obtained simultaneously from finger and from vein punctures and measured in several different ways.　Table II gives the results of some experiments in which the comparisons were made for

TABLE II.

Comparison of Simultaneous Sugar Content of Capillary Blood (Folin Ferri-
cyanide Method) and Venous Blood (Folin-Wu Method and
Folin Ferricyanide Method) after a Single Mixed Meal.

Time.	Finger blood. Folin ferricyanide method.	Venous blood.	
		Folin-Wu method.	Folin ferricyanide method.*
Experiment 1; Subject 4.			
p. m.	*mg. per cent*	*mg. per cent*	*mg. per cent*
6.00†	95	95	92
7.00	123	117	118
8 00	130	127	127
9.00	116	118	116
10.00	102	101	102
11.00	104	105	105
Experiment 2; Subject 5.			
6.00	114‡	114‡	114‡
7.00	121	103	102
8.00	115	106	104
9.00	99	88	87
10.00	103	102	96
Experiment 3; Subject 7.			
6.00	104	92	88
7.00	120	91	93
8.00	116	115	116
9.00	92	90	91
10.00	104	99	102
11.00	99	97	94

* See second foot-note (†), Table I, regarding the collection of blood for analysis by this method.

† Dinner followed immediately after the first blood sample was taken.

‡ The subject had played tennis shortly before these samples were obtained.

several hours after a single mixed meal. The data presented indicate: (1) With fasting individuals the finger blood when measured by the Folin ferricyanide method gives sugar values slightly lower than when simultaneously obtained venous blood is measured by the Folin-Wu copper reagent. (2) When the ferricyanide and copper methods are compared in their action upon the same filtrate the former gives slightly lower results. (3) When the ferricyanide method only is used the blood of fasting individuals shows substantially the same sugar content in both capillary and venous blood. For a period after a meal the sugar content of the capillary blood exceeds that of the venous blood and then both return to the same level. These comparisons reveal smaller differences in some respects than were obtained in the few preliminary figures reported by Folin (10).

Results.

The concentrations of sugar (total reducing substance) in the capillary blood of nine young men as measured at hourly intervals for an entire day is recorded in Table III. In considering this attention should be directed first to the blood sugar concentrations during that portion of the 24 hour period which the subjects spent in a state of complete rest in bed. In most instances this was from 12 o'clock midnight until 6.30 a.m. In every case the beginning and conclusion of this period is clearly marked in Table III. At first glance it is apparent that the blood sugar concentrations of all of the individuals studied falls within a limited area during this period. The mean of 62 observations upon nine individuals in a state of complete rest beginning at 6 hours after a meal is 94.48 mg. per cent ± a mean deviation[1] of 3.5 mg. per cent. The hourly averages (see the last column) range from 93 to 97 mg. per cent. If attention is focused upon the behavior of each individual singly, it will be noted that the variations are still more limited. Subjects 1, 2, 7, 8, and 9, with the mean blood sugar values 91 ±1.1, 95 ±1.7, 92 ±1.6, 95 ±1.4, and 92 ±1.7, respectively present the greatest uniformity and Subject 5, with an average concentration of 96 ±3.4, exhibits the largest fluctuations. Incidentally the small deviations found within the individual

[1] For the method of calculating the mean deviation see Scott (13), p. 87.

TABLE III.

Hourly Capillary Blood Sugar Concentrations of Normal Young Men Living upon a Normal Diet.

Subject No.	1	2	3	4	5	6	7	8	9	Average.
Time.					Sugar concentrations, mg. per cent.					
a.m.										
8.00	98	100	95	95	105	93	93	94	95	97
8 10–8 30, breakfast.										
9.00	138	140	108	140	102	118	124	127	95	121
10.00	102	92	92	99	101	92	98	108	109	99
11.00	106	112	99	105	107	93	94	94	90	101
p.m.										
12.00	105	103	112	117	99	99	99	93	93	103
12.10–12 30, luncheon.										
1.00	157	140	122	115	140	96*	99*	127	119	132
2.00	141	127	109	131	110	†	141††	106	105	118
3.00	123	132	122	129	99	126‡	107‡	110	95	113
4.00	96	106	129	96	103	105‡	105‡	110	103	103
5.00	101	92	98	98	99	93‡	97‡	97	90	96
6.00	95	96	88	108§	105§	90	106§	97	90	93
6 10–6 40, dinner.										
7.00	140	109	98	127	131	125	142	180	133	130
8.00	161	119	129	133	105	143	118	148	131	134
9.00	154	116	123	146	95	134	93	129	114	126
10.00	93	103	108	134	96	106	102‖	95	92	103
11.00	91¶	105	120	107	98	94	92	95	96	99

a m.										
12 00	94**	108	105¶	97¶**	91¶	90¶	94¶**	92	87	94
1 00	91	98¶**	99**	101	99**	96**	92	95¶	94¶	97
2 00	91		99	98	98	91	95	**	91**	94
3 00	90	96	99	103	92	93	91	97	95	96
4 00	91	94	98	96	96	95	91	94	92	95
5 00	91	95	95	97	95	88	91	94	91	93
6 00	91	96	98	97	100	91	91	96	92	95
7 00	92††	93††	99††	98††	95††	95††	90††	93	90	94

7 30–8 00, breakfast.

8 30	111	146	143	105	104	144	164	96*††	88*††	131
9 00								129††	136††	

Mean values for period of complete rest in bed.

Mean......	91	95	99	98	96	92	92	95	92	
" deviation.	1 1	1 7	3	2 6	3 4	2 8	1 6	1 4	1 7	

Mean age of subjects = 23 5 ±2.5 years.   Mean weight = 71 ±10.6 kilos.

* Excluded in computing average for this hour.
† Meal preceding this sample.
‡ Included with values for preceding hour in computing their average.
§ Exercise preceding this sample.
‖ Ate a piece of cake preceding this sample.
¶ In bed.
** Asleep.
†† Arose, took shower bath, and dressed preceding this sample.

series demonstrate that the method of drawing blood and measuring its sugar content is capable of a high degree of accuracy. Taken altogether these observations present a picture of a definite sugar level in the blood of fasting and resting individuals which is maintained over a number of hours with a high degree of constancy. Each individual seems to have his own characteristic level, which we might describe as his basal blood sugar value. These individual levels do not diverge very far from the group average of 94 to 95.

Hansen (14) has reported some studies upon the constancy of the blood sugar in fasting normal individuals. Her observations were made during the morning hours. The subjects reclined upon a bed in the laboratory—one of them is mentioned as having slept throughout most of the experiment—and numerous blood samples were taken from the ears at intervals of about 2 minutes for nearly 2 hours. The individual curves show continuous small oscillations in blood sugar concentrations, many of which are included within a range of 90 to 100 mg. per cent. At intervals, however, the curves dip to the neighborhood of 80 mg. per cent and after a variable number of observations have been obtained at that level it rises again to the 90 to 100 mg. range, or may even move more or less rapidly well into the 100 to 110 mg. per cent level for a time, and again fall back. This investigator concluded that the fasting blood sugar is not an absolutely constant quantity on the same day in the same individual but that it fluctuates between 80 and 110 mg. per cent in waves whose length is usually somewhat less than 1 hour. During the time that our subjects spent in the condition of complete rest they yielded but very few values not included by the limits 90 to 100, and those might be ascribed to variations in technique. It is possible, of course, that we have missed the extreme variations by the interval between samples, though the longer experimental period should have favored encountering some of them. It may be true also that the regulation of blood sugar concentration at night may be accompanied by fluctuations within a narrower range than is true even in a resting individual in the day.

For many purposes it does not matter greatly whether the limits of constancy of the blood sugar are defined as 90 to 100 mg. per cent or 80 to 110. To know the situation as precisely as

possible does become of importance, however, when we are attempting to determine just how large a fluctuation must be in order to be interpreted as physiologically significant.   It is usually assumed that a variation must exceed 3 times the mean deviation of a series in order that importance may be attached to it.   With our data the mean deviations in individual cases ranged from ±1.1 to ±3.7 mg. per cent, from which it would follow that variations from the mean must be greater than from 3 to 11 mg. per cent in order to have any significance.   Since the larger number is approximately 3 times the mean deviation (±3.5) for the group as a whole, it is probably safer to use it to mark the range of normal values which gives us 83 to 106 mg. per cent as including the most probable area.   Actually we doubt whether basal values for a single individual really extend over all of this region.   The frequency of the values which lie between 90 and 100 mg. per cent (as well as the tendency of the group average to return to this same territory after a meal) suggests that this constitutes the real normal zone for fasting blood sugars and that the remainder is a border-land of uncertainty due to factors not yet fully controlled.   In consideration of the limitations of present methods of sampling and analysis we have refrained from attaching significance to blood sugar values unless they lie at or beyond the boundaries just indicated.

With this as a background we may now consider the earlier portions of Table III.   At the outset it should be stated that no attempt was made to time the taking of blood samples so that the peak values after meals might be located.   Very frequent—almost continuous—samples would have been necessary to accomplish this and class schedules made such procedure impossible for us.   It will be observed that some high values—above 150 mg. per cent—were obtained but they were not numerous.   Petrén (5), the only investigator to report data at all extensive upon sugar content of capillary blood in normal people after mixed meals, does not record any value higher than 139 and he took samples every half hour for the first 2 hours.   Whether after a mixed meal high sugar concentrations are really infrequent cannot be determined until more evidence is at hand.   Undoubtedly this feature varies with the quantity of rapidly absorbable carbohydrate consumed and may be a very transitory event in daily life.

In considering the fluctuations of the blood sugar after the different meals, attention should be directed to the hourly average values as probably the more significant. The hyperglycemia due to breakfast disappeared at 10 o'clock, or about $1\frac{3}{4}$ hours after the beginning of the meal. After luncheon and after dinner the elevation of the averages persisted for more than 3 hours from the time the food intake commenced. Our results indicate that the meals of the day differ in the extent of the hyperglycemias which they provoke. According to our computation the approximate average carbohydrate intake was about 80 gm. at breakfast, 105 gm. at luncheon, and 122 gm. at dinner, which points toward considerable correlation between the carbohydrate ingested and the extent of the resulting hyperglycemia.

Foster (15) has reported four experiments in which 100 gm. of glucose were ingested and the sugar content of the capillary blood was followed afterward, by a micro modification of the Folin-Wu method (12). Averages of the values which he has recorded for the various intervals give a fairly smooth curve which indicates that after ingesting glucose alone the capillary blood returns to normal in about 150 minutes. Gilbert, Schneider, and Bock (16) have recorded a more extensive series of experiments in which a slightly larger dose (averaging 119 gm.) of glucose was given. Bock's micro procedure (17) was employed and a considerable number of blood samples were obtained at rather short intervals. The individual curves vary with respect to each other but after 150 minutes the average sugar concentration had returned to within 12 mg. per cent of the fasting value and after 180 minutes it was at approximately the original level. In our experiments the luncheons and dinners had carbohydrate contents approximately corresponding to those of Foster's and Bock's experiments respectively. Our results give some indication that with a mixed meal a slightly longer period is required for complete return to fasting blood sugar levels, though our observations were not sufficiently frequent to enable a conclusion upon this point to be reached with certainty. In this connection an observation of Cori (18) is of interest. He found that in rats the presence of an amino acid (glycine) reduced the velocity of the absorption of glucose from the alimentary tract. With the pure carbohydrate the rate was 1.80 gm. per kilo per hour, but when a

molecular equivalent of glycine was present, the absorption rate dropped to 1.02 gm. of glucose per kilo per hour. Similar data are not available for man, but it seems quite probable that a lessened rate of absorption of carbohydrate from mixed meals might obtain here as well.

Effect of Sleep.

Blood sugar measurements were obtained upon one of us while carrying on laboratory measurements throughout the night. As a mean of seven observations made between midnight and morning the average value of 101 ±4.7 mg. per cent was obtained. A few nights later the experiment was repeated on the same subject during his sleep. On this occasion the mean value of six observations was 101 ±2.5 mg. per cent. These experiments by themselves are insufficient for any conclusions and are mentioned here only in view of the close agreement which we have found between the average blood sugar concentrations of nine sleeping individuals and the average sugar content of 84 fasting individuals measured in the morning hours (see a later section).

Effect of Mild Exercise.

In the course of the principal experiments the subjects worked in the chemical laboratory during the portion of the morning from 10.00 a.m. on. They were on their feet, moving about, and carrying on various laboratory procedures until the lunch hour. This did not cause a definitely distinguishable change in the average blood sugar. In several of the individual cases the record does show a rise in sugar concentrations at 11.00 a.m. or at 12.00 p.m. which is sufficiently pronounced to exceed probable experimental errors. Unfortunately our attention was not focused upon these instances at the proper time so they were not correlated with the subjects' activities over that particular interval. Subjects 4, 5, and 7 were known to have exercised in various ways between 5.00 and 6.00 p.m. on the day of the experiment. Subject 4 played tennis a short time and came directly from the courts to our laboratory for the taking of the blood sample. Subject 5 took a short run during the earlier portion of the hour, and Subject 7 came in from a brisk walk. The increases in blood sugar for this hour were 10, 6, and 9 mg. respectively. In the first and

the third cases this augmentation is more than 3 times the mean deviation found in the basal sugar values of these individuals and may possibly be regarded as signifying an actual physiological event. However, the sugar values found after the exercise are too near to the zone of uncertainty (discussed in a previous section) for the results to be considered definitely significant.

These observations led us to follow up the matter of exercise and its effect upon blood sugar to a limited extent. In Table IV we record some of the data which we have accumulated. The change from a condition of complete rest in bed to the normal upright position and the attendant activities therewith do not

TABLE IV.

Effect of Mild Exercise upon Concentration of Blood Sugar in Fasting Individuals.

All experiments began at 7.30 a.m. Finger blood and Folin ferricyanide method used.

Subject No.	Resting in bed.	After arising and dressing.	After walking 3400 ft.	Time required for walk.
	mg. per cent	*mg. per cent*	*mg. per cent*	*min.*
A	95	96	96	15
B	92	91	91	15
C	92	94	95	15
D	99	101	99	13
E	99	98	101	13
F		100	104	8
G		104	114	8

produce any demonstrable effect upon blood sugar concentrations. Nor does walking upon a level appear to cause an increase in the capillary sugar until the rate of exercise is accelerated to such an extent that rather strenuous effort is required. The literature bearing upon this point is voluminous and conflicting. In much of it sufficient time had not elapsed after food intake to be certain that this factor was not involved. Further experiments along this line are in progress.

Normal Morning Concentration of Sugar in Capillary Blood of Fasting Young Men.

A series of observations upon the capillary blood sugar concentrations of 84 normal young men selected at random from all

four classes of the school was obtained in connection with this investigation. The samples of blood were obtained between 7.30 and 8.00 a.m. as the subjects were about to enter the dormitory dining room. The subjects had exercised only to the extent necessary to arise and dress themselves. The Folin micro method was employed. The observations are recorded in Table V. The mean concentration of blood sugar in the series of 84 was 93.6 ±4.6 mg. per cent. This corresponds very closely to the value 94.48 ±3.5 mg. per cent which was found to be the mean of the 62 observations upon nine individuals at complete rest (see Table

TABLE V.

Morning Fasting Blood Sugar Content of 84 Normal Young Men Measured by Folin Ferricyanide Method.

All values are expressed in mg. per cent. The observations are recorded in horizontal lines in the order in which they were obtained.

98	100	95	95	105	93	93	94	95	95
92	100	90	90	90	89	95	100	92	95
96	91	88	87	90	86	92	96	92	93
92	101	90	82	91	91	92	102	99	93
98	103	99	98	100	105	93	98	101	100
96	90	90	90	92	90	91	97	90	90
93	92	85	85	91	91	95	94	92	94
91	88	91	96	88	91	93	94	102	92
92	95	91	98						

Mean of this series = 93 6 mg. per cent.
    "   deviation = ± 4.6 mg. per cent.
    "   age = 22.8 ± 2.3 years.
    "   weight = 70.9 ± 7.6 kilos.

III). Each series was obtained independently and the computation of the respective means was not made until both series had been completed. The close agreement between the results obtained when repeated measurements were made upon a small carefully controlled group and when single observations were obtained upon a large group suggests that either procedure is satisfactory if the same care in the technique is exercised. In a paper which came to us after our experimental work was completed Pierce and Scott (19), using venous blood and the Shaffer-Hartmann method, obtained 97 ±7 mg. per cent as the mean of

141 measurements upon as many fasting individuals in a state of rest. They submit their data to statistical analysis and conclude that the mean value for their series did not change greatly after approximately the first 28 observations had been obtained. For a discussion of much of the earlier work upon normal concentrations of sugar in the blood reference should be made to the summary by Gray (20).

SUMMARY AND CONCLUSIONS.

The concentrations of sugar (total reducing substances) in the capillary blood of nine normal young men living upon their usual diet has been determined at hourly intervals over a period of 24 hours by the Folin micro method. In each individual subject the concentration was maintained at a very constant level while at rest in bed during the night. This level varied slightly with the individuals but in no case did it differ widely from the group average of 94 to 95 mg. per cent. This was approximately the value found also as the mean concentration of sugar in the capillary blood of a much larger group of young men in the postabsorptive condition. Therefore, it is concluded that sleep does not cause a significant change in the blood sugar concentration. There was some indication also that after mixed meals the capillary blood sugar returns to fasting levels somewhat more slowly than others have found after the ingestion of pure carbohydrate. Various forms of mild exercise did not produce any significant change in blood sugar. Only as the intensity of the exercise was increased markedly was any indication of an increase of sugar concentration obtained.

We wish to acknowledge the cheerful cooperation of the students who served as subjects for these experiments. To Mr. Maurice Dionne and Mr. Donald Simons we are indebted for some assistance in the experimental work.

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THE INFLUENCE OF DIET ON THE BODY FAT OF THE WHITE RAT.*

By H. C. ECKSTEIN.

(From the Laboratory of Physiological Chemistry, Medical School, University of Michigan, Ann Arbor.)

Some of the questions regarding the synthesis and deposition of fatty acid radicals in the animal body have recently been answered by Ellis and Isbell (1) in their thorough and painstaking study of the soft pork problem. These investigators have clearly demonstrated that the physical and chemical properties of lard are dependent upon the diet, and that the fatty acid make-up of lards is invariably like that of fatty substances in the diet. Thus arachidic acid was present in largest amounts in the "fat" obtained from hogs ingesting peanut oil, a product characterized by its high content of arachidic acid, while the amounts of the linoleyl radical predominated in the oily fats synthesized as a result of the ingestion of soy beans, which contain unusual amounts of that radical. Confirmation of this work came from the hands of Anderson and Mendel (2), who proved that the quality of the "fat" produced by the white rat varies with the diet. Animals on rations containing lipids with high iodine numbers invariably deposited lipids in their tissues with iodine numbers higher than those of the lipids isolated from rats on diets devoid of preformed "fat." Anderson and Mendel (2) also showed that the "soft fat" of an animal like the rat can easily be transformed into the so called "hard fat" by mere dietary changes. The economic significance of this important contribution certainly calls for no discussion. These investigators likewise found that, so far as the iodine number and index of refraction are concerned,

"fat" synthesized by rats on diets almost devoid of preformed "fat" or carbohydrate is very similar to that formed by rats on diets high in carbohydrate but practically free of "fat." This suggests that the type of lipids synthesized from protein is like that formed from carbohydrate.

No analyses have been reported for the fatty acids obtained from the lipids of rats fed on purely synthetic diets. The purpose of the investigation reported here is therefore to study that question and to consider two phases of it: first, to determine the fatty acid make-up of lipids synthesized by rats from "fat"-free precursors; and second to determine the effect of the ingestion of fatty substances of known composition to this make-up.

As a result of this study it has been shown that the white rat can readily synthesize palmitic, stearic, and oleic acids from diets free of those lipids. It has likewise been demonstrated that the myristyl and oleyl radicals are deposited in the tissues when they are a part of the diet. The more highly unsaturated acids, arachidonic and linoleic, are always present in rat tissues regardless of the diet ingested. The production of these acids seems to be stimulated by feeding triolein, for of the diets fed, Diet 5, which contained triolein, caused the largest deposition of the unsaturated acids. The general effect resulting from the ingestion of sodium tributyrate differed from that observed when other lipids were ingested in that a definite change in the fatty acid make-up took place without the actual incorporation of the butyryl radical in the lipids of the rat.

Procedure.

White rats weighing approximately 40 gm. were fasted for 48 hours and then placed for a period of 8 weeks on chosen experimental diets summarized in Table I. It is evident from Table I that the rations are alike so far as their contents of the yeast concentrate, Vegex, and the Osborne and Mendel salt mixture are concerned. With this exception, however, they all differ and can be divided into two distinct groups. Group A, Diets 1 and 2, was made as free of preformed "fat" as could be accomplished by a thorough extraction of the protein and starch with ethyl ether. The Vegex was not extracted with "fat" solvent but the amount of lipids present is negligible inasmuch as only

0.02 per cent of fatty substances soluble in petroleum ether could be removed from that concentrate. Some lipids necessarily became a part of the diet as the result of the addition of the non-saponifiable matter of cod liver oil, but this incorporation can be neglected as a source of fatty acids since the fraction of the oil used was free of fat and fatty acids. Diets 1 and 2 differed from each other in that the latter is free of preformed "fat" and carbohydrate, while the former, though it may be free of "fat," nevertheless contains large amounts of starch. The animals on Diet 1 thus had the opportunity of synthesizing lipids from

TABLE I.
Composition of Diets. *

	Group A. Free from preformed "fat."		Group B. Containing preformed "fat."		
	Diet 1.	Diet 2.	Diet 3.	Diet 4.	Diet 5.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Vegex	2	2	2	2	2
Salt mixture†	5	5	5	5	5
Casein	14.25	88.5	14.25	14.25	14.25
Gelatin	0.75	4.5	0.75	0.75	0.75
Starch	78		58	54	58
Myristic acid			17.6		
Sodium butyrate				19	
Triolein					20
Glycerol			2.4	5	

* The non-saponifiable matter obtained from 7 gm. of cod liver oil was added to each 100 gm. of the diets.

† Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37**, 572 (1919).

carbohydrate and small amounts of protein, while those on Diet 2 were necessarily forced to make their fatty substances from protein. Rats on diets in Group B were in a position to transform preformed "fat" into body "fat" as well as to synthesize fatty substances from "fat"-free precursors. In order to obtain more detailed information concerning this question, it was thought advisable to analyze fractions of the animals rather than merely to characterize the lipids of the whole rat. The individual bodies of the animals were therefore divided into several fractions. Fraction 1 consisted of the hides of the animals, Fraction 2 of

all the organs except the brains (the "fat" adhering to the organs was included in this fraction), Fraction 3 of the brains, and Fraction 4, which is termed carcass, of the residual parts of the animals. The gastrointestinal tract was always discarded.

The total lipids were removed from the fractions according to a procedure previously described (3). Briefly stated the method consisted of exposing the material to be extracted first to the vapors of absolute alcohol, then to chloroform vapors, and finally to the vapors of ethyl ether. The combined extracts were concentrated *in vacuo* and the lipids removed from the concentrate by extraction with petroleum ether (b.p. 20–40°). Tests made on the distillates obtained during the concentration showed that no fatty acids were lost during the evaporation of the various solvents.

Sufficient material was not available for the separate analysis of the lipids isolated from the different fractions of each rat, and combinations of similar fractions were therefore necessary. In the case of the hides and the carcasses, mixtures of the lipids obtained from two rats were always made, while in the case of the brains and the organs, composites were made of all the fractions secured from a particular group of rats. Even though a composite was made in the case of the lipids obtained from the brains, enough material was obtained for total lipid and cholesterol determinations only. In the other cases, however, detailed analyses were possible, and as a result of these, data are given showing the iodine number, the saponification number, and the percentages of cholesterol, arachidonic, and linoleic acids.[1] The saturated acids were separated from the unsaturated ones by Twitchell's method. This procedure invariably resulted in losses of fatty acids. This is in line with Bloor's observations (4) and may, as he has pointed out, be due to a destruction of some of the constituents during the separation. In some cases the distribution of the acids in the saturated fractions was determined. This was accomplished by making and fractionally distilling the methyl esters of these saturated fatty acids. In order to give some basis for determining whether some of the substances found in the adult ani-

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[1] Iodine numbers were determined by the Hanus method, cholesterol by the digitonin method, and the more highly unsaturated acids by the well known bromination method.

TABLE II.

Total Lipids and Highly Unsaturated Fatty Acids in Carcasses of Rats on Various Diets.

Type of diet	Carbo-hydrate.	Protein.	Myristic acid.	Sodium butyrate.	Triolein.	Control.*
Diet No.	1	2	3	4	5	6
No. of rats on diet	6	6	4	6	6	24
Average weight of rats, gm.	204	121	173	120	139	40
" " carcasses, gm.	113	54	101	63	81	22
" content " total lipids, gm.	14.15	3.98	15.0	4.46	6.0	1.2
" " arachidonic acid, gm.	0.022	0.013	0.019	0.013	0.028	0.015
" " " linoleic	0.0452		0.0332		0.072	0.0339
Arachidonic acid in carcass, per cent	0.02	0.02	0.02	0.02	0.03	0.07
Linoleic " " " "	0.04		0.03		0.09	0.25
Total lipids " " "	12.5	7.44	14.9	7.13	7.4	5.4
Arachidonic acid in total lipids, per cent	0.15	0.31	0.13	0.29	0.47	1.24
Linoleic " " " "	0.32		0.22		1.19	2.80

* Young rats, see text.

TABLE III

Total Lipids and Highly Unsaturated Fatty Acids in Hides of Rats on Various Diets

Type of diet	Carbo-hydrate	Protein	Myristic acid	Sodium butyrate	Trolein	Control *
Diet No	1	2	3	4	5	
No. of rats on diets	6	6	4	6	6	24
Average weight of rats, *gm*	204	121	173	120	140	40
" " hides, "	36 7	20 2	31 2	20 4	24 1	5 8
" content " total lipids, *gm*	11 82	4 97	9 15	5 2	5 63	0 76
" " " arachidonic acid, *gm*	0 010	0 0086	0 0066	0 0068	0 0092	0 0034
" " " linoleic " "	0 0269		0 0137		0 0266	0 0119
Arachidonic acid in hides, *per cent*	0 02	0 04	0 02	0 03	0 04	0 05
Linoleic " " " "	0 06		0 04		0 10	0 35
Total lipids	32 2	24 6	29 3	25 1	23 5	13 1
Arachidonic acid in total lipids, *per cent*	0 07	0 17	0 07	0 13	0 16	0 44
Linoleic " " " "	0 21		0 15		0 47	2 04

* Young rats, see text

TABLE IV.

Total Lipids and Highly Unsaturated Fatty Acids in Organs (Less Brain) of Rats on Various Diets.

Type of diet	Carbo-hydrate.	Protein.	Myristic acid.	Sodium butyrate.	Triolein.	Control.*
Diet No.	1	2	3	4	5	
No. of rats on diet.	6	6	4	6	6	24
Average weight of rats, gm.	204	121	173	120	139	40
" " " organs, "	21.7	16.4	14.8	14.9	13.7	5.82
" content " total lipids, gm.	4.4	1.63	1.49	1.79	1.34	0.204
" " " arachidonic acid, gm.	0.0054	0.0057	0.0050	0.0071	0.0111	0.0045
" " " linoleic "	0.0218		0.0285		0.0311	0.0156
Arachidonic acid in organs, per cent.	0.02	0.04	0.03	0.04	0.08	0.08
Linoleic " " " "	0.09		0.19		0.22	0.26
Total lipids " " " "	20.2	9.99	10.1	12.05	9.77	3.5
Arachidonic acid in total lipids, per cent.	0.11	0.36	0.34	0.40	0.82	2.23
Linoleic " " " "	0.46		1.19		2.26	7.62

* Young rats, see text.

TABLE V

Total Lipids and Highly Unsaturated Fatty Acids in Whole Rat (Less Brain and Gastrointestinal Tract)

Type of diet	Carbo-hydrate	Protein	Myristic acid	Sodium butyrate	Triolein	Control *
Diet No	1	2	3	4	5	24
No. of rats on diet	6	6	4	6	6	40
Average weight of rats, gm	204	121	173	120	139	40
" " material analyzed, gm	172	147	119	90	98	33
" content " total lipids, gm	30 4	10 6	25 6	11 5	13 0	2 2
" " " arachidonic acid, gm	0 0374	0 0273	0 0306	0 0269	0 0443	0 0229
" " " linoleic " "	0 0939		0 0754		0 1297	0 0697
Arachidonic acid in material analyzed, per cent	0 02	0 03	0 02	0 03	0 04	0 07
Linoleic " " " " "	0 06		0 05		0 11	0 21
Total lipids	17 7	11 7	17 4	11 7	10 9	6 6
Arachidonic " total lipids, per cent	0 12	0 26	0 12	0 23	0 37	1 06
Linoleic " " " " "	0 31		0 30		1 00	3 21

* Young rats, see text

mals were actually synthesized by these animals or whether they were substances already present at the time when they were placed on the experimental diets, rats weighing 40 gm. were fractionated like the adults and complete analyses were made of the lipids of these fractions.

For ease of comparison and for the sake of conserving space, the results tabulated are given in terms of an individual rat. Thus in the case of Diet 1, when six rats were used, the sum of the total lipids and their constituents is divided by 6, while in the case of Diet 3, when only four animals were used, the sum is divided by 4.   Similar calculations were made for the other groups.

TABLE VI.

Iodine and Saponification Numbers of Lipids Obtained from Rats on Various Diets.

Source of lipids.	Diet 1.		Diet 2.		Diet 3.		Diet 4.		Diet 5.		Con- trol.*
	Iodine No.	Saponification No.	Iodine No.	Saponification No.	Iodine No.	Saponification No.	Iodine No.	Saponification No.	Iodine No.	Saponification No.	Iodine No.
Carcasses....................	67	198	64	192	47	207	53	196	77	194	81
Hides.......................	65	197	62	193	48	205	51	196	76	196	80
Organs......................	72	198	74	194	53	211	56	202	80	195	88

* Young rats, see text.

In Tables II to V are shown the average weights of the animals as well as the average weights of the fractions obtained from them.   Similar results are added for the young rats.   A study of these data shows that, with the exception of the rats on Diets 1 and 3, the growth rate was not very good.   The fact that the animals scattered their food made it impossible to make accurate measurements of food intake.   It was, nevertheless, evident merely from observations that those animals that did not grow very well ate the least amount of food.   This is probably the explanation of the variation in the rate of growth.   In spite of this fact, there is no good reason to believe that the differences

noted for the lipids of the rats on the various diets are due to a variation in the growth curve of the animals. Anderson and Mendel (2) have observed a decrease in the iodine numbers of the lipids of rats with increases in the body weight of the animals. In the experiments reported here age cannot be a factor, since, with the exception of the young rats, the animals were all killed at approximately the same age. A comparison of the iodine numbers in Table VI indicates that body weight likewise plays no rôle in determining the nature of the lipids. Thus the iodine numbers of the fatty substances obtained from the animals on Diet 1 coincide with the iodine number of the animals ingesting Diet 2 in spite of the fact that the former grew at a much better rate than did the latter. If carbohydrate is an intermediate in

TABLE VII.

Composition of Saturated Fatty Acid Fraction of Lipids Obtained from Rats on Diets 1 and 3.

Source of lipids.	Diet 1.			Diet 3.		
	Myristic acid.	Palmitic acid	Stearic acid.	Myristic acid.	Palmitic acid.	Stearic acid.
	per cent	per cent	per cent	per cent	per cent	per cent
Carcasses............	?	26 4	12.2	15.4	19.9	8.6
Hides	?	24.5	11.4	17.6	16.4	6.9

the conversion of protein to fat, small, if any, differences should be detected between lipids formed by the rats on Diets 1 and 2. The iodine number of the fatty substances of rats ingesting a diet rich in triolein is higher than that of any other group of adult animals. On the other hand those lipids obtained from rats ingesting sodium butyrate and myristic acid had the lowest iodine numbers. The animals on the triolein and sodium butyrate diets grew at a rate quite similar to that of the animals ingesting a diet which consisted mainly of protein, while those of the myristic acid group grew at a rate much like that of the group on Diet 1. These facts therefore justify the above contention, that the differences in the iodine numbers cannot be ascribed to a variation in the rate of growth or to the body weight of the individual animals and must be due to the type of diet ingested.

Most of the results shown in Table VI are in line with the current view that the nature of the body lipids is determined by the nature of the diet. Thus the iodine numbers of the lipids obtained from animals on the diet high in carbohydrate, but free of preformed lipids, are like that found for the lipids of animals on a diet containing much protein and neither preformed fat nor carbohydrate. Myristic acid is a saturated lipid with a molecular weight that is lower than the mean molecular weight of the fatty acids predominating in the tissues of the animals on fat-free diet. When fed to rats a drop in the iodine number and a slight, but significant, rise in the saponification number of the total lipids took place. That the myristic acid radical was actually deposited in the tissues of the animals ingesting it is to be seen from the data in Table VII, where the amounts of the saturated fatty acids isolated from the lipids of the animals on Diets 1 and 3 are given. The determinations were made by fractionally distilling the methyl esters of the saturated acids. Repeated fractionations were made so as to assure the presence of no more than two constituents in each fraction. The free fatty acids were then regenerated from the esters and the mean molecular weight of each fraction determined by titration. The constituents making up each fraction were calculated from the weight of the fraction and its mean molecular weight. It will be observed from Table VII that large amounts of the myristic acid radical were deposited when a diet containing myristic acid was fed. The ingested acid apparently supplants some of the palmitic and some of the stearic acid, but it must also displace some of the oleic acid since the amount deposited is greater than can be accounted for by the sum of the decrease of the other two acids, and furthermore since the iodine number of the total lipids is less than that found for animals on Diets 1 and 2. The presence of myristic acid in the tissues of rats that did not ingest it is questionable. It is likely that small amounts are present, since a small fraction having approximately the same boiling point as methyl myristate was obtained from the fatty acid isolated from animals on Diet 1. Sufficient amounts were, however, not available for purification and for further study so that the presence of the acid must therefore be questioned.

The effects obtained by feeding sodium butyrate were not

wholly expected. As a result of other observations a drop in the
iodine number and a greater increase in the saponification number
than that which took place when myristic acid was fed was looked
for. Only the first part of the prediction proved to be true. This
is evident from the results recorded in Table VI, where it is to be
seen that, although low iodine numbers were obtained, the
saponification number, with the exception of that of the lipids
of the organs, remained quite constant. The slight increase noted
for the lipids in the organs is not as high as would be expected
in the light of the results obtained when the myristic acid was
fed. That the animals were not entirely normal was evident from
the fact that they urinated so frequently that it was impossible
to raise them under ideal conditions even though the cages were
cleaned every day. Sodium butyrate is of course quite alkaline
and this may explain the polyuria, but whether the alkalinity
causes the unlooked for effect on the fatty acid make-up has not
been determined. Attempts to introduce the butyryl radicals
by other means have thus far failed. Glyceryl tributyrate, as
well as di- and monobutyrates of glycerol can, on account of
their intense bitterness, not be used. Diets containing as little
as 5 per cent of tributyrin were invariably refused by the ani-
mals. Butyric acid was likewise found to be an impossible in-
gredient of the diet. Confirmation is, however, very essential
and further attempts are now being made along these lines.

The data tabulated in Tables II to V are chiefly concerned
with amounts of the unsaturated fatty acids, arachidonic and
linoleic, present in the lipids of the different rats. In order to
throw light on the ability of the animals to synthesize these acids
the results should be compared with those of the lipids of a group
of rats each weighing approximately 40 gm. These animals were
fasted for 48 hours, then killed, and separated into the same
fractions as obtained in the adult rats. It will be recalled that
the rats placed on the diets weighed approximately 40 gm. at
the beginning of the experimental period.

The data are in accordance with those reported by Anderson
and Mendel (2) for they show that the lipids of young rats differ
from those present in more mature ones in that the total lipids of
the tissues in the former contain larger quantities of the more
highly unsaturated acids than are present in the older rats. ·The

data are also in accordance with the prevailing view that the lipids in the organs contain more of the unsaturated acids than do the lipids of other parts of the body. With respect to actual amounts, however, the carcasses invariably contain more of the highly unsaturated acids than do any of the other fractions. Calculated in terms of per cent of the total arachidonic acid, from 50 to 60 per cent was found to be in the various carcasses, only 15 to 23 per cent in the organs, and from 20 to 30 per cent in the hides. In other words more than one-half of these unsaturated compounds appears in the carcasses and approximately as much is present in the hides as in the organs. This distribution is, as can be seen from Tables II to V, independent of the diet. On the other hand the distribution of the total lipids varies with the diet. Thus while the actual amounts of these predominate in the carcasses of animals on Diets 1, 3, and 5, slightly greater amounts are to be found in the hides of those animals on Diets 2 and 4. The percentage of the total lipids is more constant in the hides than in the carcasses. It appears from these results that the failure to put on "fat" is more noticeable in the carcasses than in any of the other fractions, for of the three fractions the carcasses appear to suffer more so far as their lipid content is concerned. The amounts of the more highly unsaturated fatty acids do not decline in proportion to fall in total lipids and it must follow therefore that the failure to grow is reflected more in the content of fatty acids like palmitic, stearic, and oleic than in the acids like linoleic and arachidonic. That this is the case is evident from the fact that while the total lipids varied from 30 to 11 gm. for those animals on Diets 1 to 4 the amounts of arachidonic acid varied only from 37 to 27 mg.

The feeding of triolein is followed by a slight, though significant, increase in the arachidonic acid content of the tissues. The average amount present in the rats on diets free of "fat" was 0.0306 gm. as compared with 0.0483 gm. for those ingesting triolein. Similarly an increase of from 0.897 to 0.1297 gm. was found for linoleic acid. An analysis of the triolein fed failed to show even a trace of the more highly unsaturated acids. Why the ingestion of triolein should call forth an increase in the formation and deposition of the other unsaturated acids is at present not clear. The rats on each diet certainly must have

TABLE VIII.

Cholesterol Content in Fractions Obtained from Rats on Various Diets.

Diet No.	1	2	3	4	5	Control.*
Average content of cholesterol in carcasses, gm.	0.1209	0.0461	0.0986	0.0619	0.0904	0.0301
"　　"　　"　　hides, gm.	0.0857	0.0431	0.0817	0.0427	0.0489	0.0183
"　　"　　"　　organs, "	0.0471	0.0329	0.0402	0.0312	0.0398	0.0077
"　　"　　"　　brains, "	0.0239	0.0236	0.0282	0.0255	0.0260	0.0017
"　　"　　"　　whole animal, gm.	0.2776	0.1457	0.2487	0.1358	0.2051	0.0587
Cholesterol in carcasses, per cent.	0.11	0.09	0.10	0.10	0.11	0.14
"　　hides, per cent.	0.22	0.20	0.26	0.20	0.21	0.31
"　　organs, "　　"	0.20	0.20	0.27	0.20	0.28	0.12
"　　brains, "　　"	1.45	1.34	1.57	1.65	1.89	1.31
"　　whole animal, per cent.	0.16	0.160	0.17	0.15	0.19	0.170
"　　lipids of carcasses, per cent.	0.85	1.08	0.66	1.39	1.50	2.58
"　　"　　hides, per cent.	0.73	0.92	0.89	0.82	0.86	2.4
"　　"　　organs, "　　"	0.94	2.02	2.70	1.75	2.89	3.77
"　　"　　brains, "　　"	24.2	19.4	20.6	21.3	22.4	20.2
"　　"　　the whole animal, per cent.	0.90	1.36	0.97	1.17	1.56	2.55

* Young rats, see text.

contained considerable amounts of the oleyl radicals in their tissues since the small content of the other unsaturated acids cannot account for the per cent of halogen absorbed by the total lipids. While no attempts were made to determine the actual amounts of oleic acid, indirect evidence shows an increase in these amounts in the lipids of the rats on Diet 5. The iodine number of those lipids was higher than that of any of the others and, while some of the increase in the amount of halogen absorbed was of course due to increments in the linoleic and arachidonic acid content, that increase is insufficient to account for the total rise in iodine number. These animals differ from the others therefore in that larger amounts of the oleyl radicals are present in the tissues. They also differ in another way, since as the result of the ingestion of food the blood of rats on Diet 5 must have contained larger amounts of oleic acid than did the circulating fluid of the other animals. Which of these factors is the causative one cannot be ascertained from the data presented.

The distribution of cholesterol in the various fractions of the rats (Table VIII) simulates that of arachidonic and linoleic acids since the carcasses invariably contain the largest amounts of the substances in question. This is, however, as far as the likeness goes, since the hides invariably contain more of the sterol than the organs, while in the case of the acids the amounts found in the hides were quite like those obtained from the organs. The difference between hides and organs disappears when the results are calculated as per cent of the tissues, for then both contain 0.22 per cent of cholesterol. On this same basis the carcasses have 0.10 per cent of the substance, the brains 1.58, and the whole animals 0.16. As can be seen from Table VIII the percentage of cholesterol in the whole animal, as well as in the various fractions, is independent of the diet. Variations occur when the calculations are made on the basis of total lipids; but these go hand in hand with variations of the total lipid contents. When the latter are high, the former are low, thus accounting for the constant results when the results are figured in the other manner. This accounts for the apparent anomaly of the organs of the animals on Diets 1 in which only 0.94 per cent of the sterol is present in the total lipids, while more than twice as much is reported for the lipids in the organs of rats in other animals.

It follows from these data that the failure of the animal to fatten is reflected in fatty substances other than cholesterol. In this respect arachidonic acid is like cholesterol since a similar constancy was observed for the acid. These results are in line with Bloor's (5) conception that certain fatty substances should be considered as essential constituents of living tissues while others are put to immediate use for energy. The writer does not intend to convey the idea that arachidonic acid itself is an essential constituent of the cell but suggests that it may be contained in the molecule of a fatty substance which, like cholesterol, is required for cellular activity.

SUMMARY.

1. Lipids obtained from rats on diets devoid of preformed "fat" were analyzed and compared with those obtained from animals ingesting fatty substances of known composition.

2. Lipids obtained from animals on a diet consisting almost entirely of protein were similar to those secured from animals on diets containing the usual amounts of protein, large amounts of carbohydrate, but free of preformed "fat."

3. A definite change in the nature of the lipids was obtained as the result of the ingestion of myristic acid, triolein, and sodium butyrate. The butyryl radical was apparently not deposited in the tissues, while the other two were.

4. Arachidonic and linoleic acids were always present in the tissues of the rat, and while the contents of the total lipids were found to vary considerably with the diets the amount of more highly unsaturated acids was appreciably altered only by the feeding of triolein.

5. The percentage of cholesterol in the rats was found to be constant and independent of the diets used.

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PHOSPHOCREATINE.

By CYRUS H. FISKE and YELLAPRAGADA SUBBAROW.[*]

(*From the Biochemical Laboratory, Harvard Medical School, Boston.*)

Elsewhere we have shown that in the resting voluntary muscles
of the cat the greater part of what was for many years regarded as
inorganic phosphate is in reality a labile organic compound
containing phosphoric acid and creatine in equimolecular propor-
tions.[1] The existence of this substance was first recognized from
the following properties: (1) it is hydrolyzed in trichloroacetic
acid muscle filtrates at an initial rate of about 25 per cent an hour
at room temperature, and 20 or more times as rapidly in the
presence of molydate,[2] and (2) it can be precipitated by various
reagents which do not precipitate free creatine, *e.g.* by copper
acetate in the presence of a slight excess of alkali.[3] On the basis
of these properties it was possible to show that the compound is
hydrolyzed during muscular contraction and resynthesized during
the recovery period.[1]

In the analysis of muscle filtrates by the colorimetric method for inor-
ganic phosphate, the complete hydrolysis of the creatine-phosphoric acid
compound at room temperature consumes about 30 minutes, during which
time the color intensity progressively increases. Delay in attaining the

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[*] Fellow of the Division of Medical Education, Rockefeller Foundation.

[1] Fiske, C. H., and Subbarow, Y., *Science*, **65**, 401 (1927); *J. Biol. Chem.*,
74, p. xxii (1927).

[2] The designation "labile phosphorus" refers, arbitrarily of course, to
that fraction of the total phosphorus in muscle which has this special
property.

[3] Precipitates containing creatine and labile phosphorus in the ratio of
1 molecule to 1 have been obtained from muscle filtrates by copper, calcium,
barium, lead, zinc, and silver in more than a dozen different combinations.
(Phosphocreatine is carried down when a precipitate of zinc hydroxide is
formed in the solution, and can be extracted from the zinc precipitate with
alkali, though less readily than in the case of copper.)

full blue color in this method is by no means confined to the analysis of muscle, but is encountered under all sorts of conditions, and usually is caused by the inhibiting action of foreign substances added in the course of preliminary treatment (excess acid, oxalate, citrate, various inorganic salts, etc.).[4] In the description of our method the device of taking two or more successive readings at intervals of several minutes was recommended as a means of detecting the presence of such interfering substances.[4] A statement was however made to the effect that the analysis should be rejected if the reading is not constant within 10 minutes, and among the procedures which we suggested might be used when the reaction is delayed to that extent was the very simple one of increasing the velocity by warming the solution. Lohmann and Jendrassik,[5] in Meyerhof's laboratory, finding also that the color develops slowly in the case of muscle filtrates, and assuming the presence of some interfering substance to be responsible, adopted the use of heat (as suggested by us earlier) under the guise of a new modification of the method. Since Lohmann and Jendrassik's interpretation of the delayed reaction is not correct their criticisms of our technique are altogether pointless.[6]

Eggleton and Eggleton,[7,8] using the Briggs method—which we have shown to be inaccurate[4]—likewise have observed the slow development of color in the case of filtrates from frog muscle, as well as the above mentioned effects of stimulation and recovery. These authors however, also without experimental evidence, chose the alternative assumption; viz., that the cause of the phenomenon is the presence of an unstable organic compound of phosphoric acid, and hazarded the guess that they might be dealing with a new variety of hexosemonophosphate,[7,8] or a "phosphoric acid ester of glycogen."[8] Somewhat later, in a paper published several months after we had announced that the labile phosphorus in muscle is combined with 1 equivalent of creatine, Eggleton and Eggleton claimed to have *shown* that muscle contains a hexosemonophosphate with the properties which have been described above, though admitting that "some doubt attaches to the nature of the hexose"[9] As a fitting accompaniment to this extraordinary assertion, for which they could not possibly have had the slightest evidence, Eggleton and Eggleton offered an elaborate hypothesis in which the attempt was made to incorporate this hexosemonophosphate

[4] Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).

[5] Lohmann, K., and Jendrassik, L., *Biochem. Z.*, **178**, 419 (1926).

[6] The only essentially new thing in the particular form of the method proposed by Lohmann and Jendrassik is the use of twice as much of the molybdate-sulfuric acid reagent as was prescribed by us. No reason is given for this change, which is an unfortunate one inasmuch as the only effect it has, as far as we have been able to determine, is to accentuate the delay in the presence of interfering substances.

[7] Eggleton, P., and Eggleton, M. G., *Nature*, **119**, 194 (1927).

[8] Eggleton, P., and Eggleton, G. P., *Biochem. J.*, **21**, 190 (1927).

[9] Eggleton, P., and Eggleton, G. P., *J. Physiol.*, **63**, 155 (1927).

into the series of chemical reactions (involving glycogen, lactic acid, *etc.*) already known to occur during muscular contraction. Needless to say the theory, based as it was on a structure devoid of facts, is valueless, and has since been quietly abandoned by its authors.

Soon after the publication of our first report[1] a small amount of phosphocreatine[10] (or creatine phosphoric acid) was isolated in the form of a crystalline barium salt.[11]   While it was therefore possible to say that a phosphoric acid compound containing creatine and no other organic substance can be obtained from muscle, the status of the creatine compound in living muscle was still far from being settled, for the yield of the pure barium salt was equivalent to only 2 or 3 mg. of phosphorus per 100 gm. of muscle, and accounted for only about 5 per cent of the labile phosphorus found in muscle filtrates.   Further work was consequently called for in order to determine whether phosphocreatine really occurs as such in nature or is merely a decomposition product.

In the meantime investigation of the physicochemical properties of pure phosphocreatine has brought out certain facts which make the matter of yield a point of paramount importance.   For example, the second dissociation constant has been found to be about 2.5×10^{-5}, or much greater than the second constant of o-phosphoric acid,[11] consequently the hydrolysis of phosphocreatine during muscular contraction would be accompanied by the liberation of a relatively large amount of base.[12]   But if the substance in the living tissue is in the form of some more complex molecule with different acid properties, physiological deductions based on the properties of phosphocreatine itself would be unwarranted.   Similar considerations apply to the interpretation of heat measurements.

The liberation of inorganic phosphate in acid solution takes place at *approximately* the same rate in muscle filtrates and in pure phosphocreatine solutions of the same acidity.   The agreement is still better when the hydrolysis is catalyzed by molybdic acid, in fact it is hardly possible to detect any difference between

[10] By the term phosphocreatine we mean now a specific substance (creatine phosphoric acid) containing 1 molecule each of creatine and phosphoric acid, and nothing else.

[11] Fiske, C. H., and Subbarow, Y., *Science*, **67**, 169 (1928).

[12] Further discussion of this point will be found below.

muscle filtrates and artificial mixtures of phosphocreatine and inorganic phosphate when the two are analyzed by the usual technique for the determination of inorganic phosphate, the color intensity being plotted against time (*vide infra*). These facts however do not establish the identity of phosphocreatine with the natural product, for one or more additional constituents would not necessarily affect the velocity of hydrolysis by acid to a significant extent.

It is, we think, not out of place at this point to give an illustration of the futility of presumptive evidence by citing an example taken from a related field. We have in mind the checkered history of lactacidogen, regarded at first by the originator of the term (Embden) as synonymous with hexose-diphosphate. The lactacidogen content of muscle is determined by the now well known procedure of incubating the hashed tissue for about 2 hours in the presence of sodium bicarbonate, and measuring the amount of inorganic phosphate set free during this process. The supposed identity of lacta-cidogen with hexosediphosphate was based primarily upon the fact that under some conditions equimolecular amounts of lactic and *o*-phosphoric acids were found to be produced during the incubation. In the course of a decade this supposition, which in the meantime had become generally accepted as correct, was abandoned, for Embden himself came to the con-clusion that normal muscle contains no hexosediphosphate whatsoever. A previously unknown form of hexosemonophosphate was discovered how-ever at the same time, and Embden at once adopted the substitute hypoth-esis that lactacidogen and this new ester are one and the same thing; in fact he regards this not as a theory at all, but as established fact. The most obvious defect in this new suggestion is that the amount of hexose-monophosphate that can be isolated from muscle by Embden's method is equivalent to only a small fraction of the lactacidogen. It is easy to say that the major portion of the substance is lost in the rather complicated process of isolation, but the hypothesis is nevertheless wrong. Although the lactacidogen phosphorus of cat muscle averages about 50 mg. per cent,[13] and although the calcium salt of hexosemonophosphate is readily soluble in water, we have found that calcium hydroxide precipitates from the neutralized trichloroacetic acid filtrate all but 20 to 25 mg. (per 100 gm. of muscle) of the organic phosphorus which is not combined with creatine. Moreover, a large part of this 20 to 25 mg. can be precipitated by reagents which leave hexosemonophosphate in solution. From present indications, therefore, not much more than one-fifth of the lactacidogen in cat muscle can be hexosemonophosphate.

As further evidence pointing in the same direction, another product has been obtained from mammalian muscle which liberates *o*-phosphoric acid

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[13] See *e.g.*, Cuthbertson, D. P., *Biochem. J.*, **19**, 896 (1925).

when incubated with hashed muscle under the conditions of the lactacidogen determination.[14]   The barium salt of this compound is soluble in water, and does not reduce copper in alkaline solution.

Finally it should be noted that voluntary muscle contains still another product with the properties of lactacidogen.   This substance is slowly hydrolyzed when the trichloroacetic acid filtrate is allowed to stand at room temperature, and according to Lohmann[15] is pyrophosphate.

The question of the identity of the labile phosphorus in muscle can actually be settled in only one way; *viz.*, by the isolation of the substance on something approaching a quantitative basis.   Accordingly the first purpose of the present paper is to give the results of our experiments on isolation designed with special reference to the yield.

Owing to the instability of phosphocreatine in living muscle it is impossible, even by the use of liquid air, to avoid partial decomposition during the preparation of the protein-free filtrate. Whether all the muscles of the hind leg, for example, are dissected individually or whether blocks of tissue are cut away without discrimination makes little or no difference.   Stimulation is inevitable in either case, and the result is that in preparations made from several hundred gm. of muscle about one-fourth of the labile phosphorus disappears at the very outset.[16]   The most that can be expected under such conditions is a protein-free filtrate containing the equivalent of approximately 40 mg. of labile phosphorus per 100 gm. of tissue.   The yield from this point on, while only about 5 per cent in the earlier experiments with barium, has now been increased to 70 per cent.[17]   It is moreover possible to say that, practically speaking, all the labile phosphorus in the protein-free filtrate is in the form of phosphocreatine, for the

[14] Fiske, C. H , and Powers, H. H., unpublished experiments.

[15] Lohmann, K., *Naturwissenschaften*, **16**, 298 (1928).

[16] An exact estimate of the loss through enzyme action is hardly possible. Because of the connective tissue, *etc.*, mixed with the muscle when it is dissected out *en masse* the sum of the inorganic phosphate and phosphocreatine is only about 80 mg. per cent (as phosphorus).   On the basis of our analyses of single muscles we accordingly assume that if the same material could be prepared without excessive stimulation the filtrate would contain about 55 mg. of labile phosphorus per 100 gm. of tissue.

[17] *I.e.* about 28 mg. of phosphorus from an amount of filtrate equivalent to 100 gm. of muscle.   The actual yield from 100 gm. of muscle is somewhat less, since it does not pay to wash the protein precipitate.

30 per cent loss experienced in the case of muscle filtrates can be traced to hydrolysis and incomplete precipitation, and is not materially smaller in control experiments with muscle filtrates to which a known amount of pure phosphocreatine has been added after the preformed phosphocreatine has been destroyed with acid and the inorganic phosphate removed by precipitation as the calcium salt. Thus in two such experiments in which 40 mg. of phosphocreatine phosphorus per 100 gm. of muscle were added the recoveries were 70 and 75 per cent, respectively.

In the only method of isolation we have been able to devise in which the yield is satisfactory the entire separation of the phosphocreatine is based upon the properties of the calcium salt, which for this purpose has two special virtues. In the first place, among the calcium salts of the phosphorus compounds found in voluntary muscle, it occupies an intermediate position with respect to solubility, a fact which permits the use of a scheme of fractionation that is neither very wasteful of material nor so elaborate as to be impracticable.

The second marked advantage of the calcium salt lies in the ease with which it can be made to crystallize without resorting to the use of heat. Phosphocreatine is so rapidly destroyed under all sorts of conditions[18] that the choice of methods for its isolation is seriously limited. No sharp separation from purely aqueous solutions can be made without the introduction of reagents which accelerate its decomposition. The consequence is that the process of isolation must depend, in part at least, upon precipitation of one or another of the more stable salts (notably those of the alkaline earth metals) by means of some organic solvent. The corresponding salts of other organic phosphoric acid compounds are likewise readily precipitated by alcohol, etc., almost without exception, however soluble they may be in water. Moreover, the separation of two or more such products along the indicated lines

[18] The catalytic action of acid and of certain heavy metals can readily be demonstrated. Decomposition by mercury and silver is particularly rapid, which is not surprising in view of the fact that creatine itself is oxidized by salts of these two metals. Extensive destruction has however been so frequently encountered unexpectedly as to lead us to believe that the stability of phosphocreatine is affected also by a number of unknown agents.

is peculiarly ineffective as long as the material comes down in the amorphous form, owing to the tendency of one salt in its precipitation to carry down others by adsorption.   As we have frequently had occasion to observe, crude amorphous salts of phosphocreatine may under some conditions be reprecipitated several times, even fractionally, without detectable change in composition.   On the other hand, once the substance has been obtained in crystalline condition, provided that the crystals do not form too rapidly, these difficulties promptly disappear.

Of the dozen or more metals which we have used in experiments on the isolation of phosphocreatine, calcium alone has given a stable salt crystallizing at room temperature.   An abbreviated account of our experience with some of the other precipitating agents tried may however be of interest in connection with the properties of phosphocreatine, and will therefore be included here before a detailed description of the isolation as the calcium salt is undertaken.   (The method of preparing the protein-free filtrate will also be given later.)

Copper.

Copper is one of the few reagents which will precipitate phosphocreatine completely without rapidly destroying it, and at the same time leave all the free creatine dissolved.   For this reason it was selected as the most suitable means of proving indirectly that muscle contains a compound of creatine and phosphoric acid.[1] While this method is no longer being used for purposes of isolation, chiefly because of the mechanical difficulties associated with large scale operations, the peculiar properties of the copper compound are of interest.

Copper is a more or less general precipitant for organic phosphorus compounds, but in the presence of an excess of alkali—as used *e.g.* by Embden[19] for the separation of carbohydrate esters and adenylic acid—it leaves the phosphocreatine in solution, or at the most precipitates only a small fraction.   If copper is to be used in the initial step of the isolation, the method which has been the most successful in our hands consists first in precipitating the phosphocreatine, along with virtually all the other phosphoric

[19] Embden, G., and Zimmermann, M., *Z. physiol. Chem.*, **167,** 114, 137 (1927).

acid compounds present, by the addition of copper acetate and only enough sodium hydroxide to make the mixture very slightly alkaline. From this precipitate it is possible to obtain a solution containing most of the phosphocreatine by extraction with sodium hydroxide. If the alkalinity of the extracting medium is properly adjusted most of the residual organic phosphorus[20] and all the inorganic phosphate are retained in the precipitate. The following experiment will show how effective this separation is on a small scale.

To 50 cc. of neutralized muscle filtrate containing 6.65 mg. of total phosphorus, including 2.48 mg. present as phosphocreatine, were added 5.5 cc. of approximately N NaOH and 10 cc. of a 6 per cent solution of copper acetate. After 5 minutes centrifugation at 3000 R.P.M. the supernatant fluid was poured off, and the sediment stirred up with 50 cc. of 0.03 N NaOH. (Weaker alkali takes out less phosphocreatine, while solutions of higher alkalinity extract a larger proportion of impurity.) The suspension was then filtered with suction and the precipitate washed with 10 cc. of water. The combined filtrate and washings contained 2.29 mg. of phosphocreatine P and only 0.17 mg. of phosphorus in other forms.

The success of this precipitation depends upon the proper adjustment of the proportion of copper acetate to sodium hydroxide. The important point is to have the supernatant fluid only slightly alkaline (pH between 7 and 8). With any given samples of reagents the correct proportion is readily determined by preliminary tests.

Determination of Creatine in Copper Precipitate.

The method of copper precipitation given above is identical with that used in demonstrating the existence of a creatine-phosphoric acid compound in the voluntary muscle of the cat.[1] For this purpose the washed copper precipitate is suspended in water, decomposed with H_2S, and the excess H_2S removed by aeration. After the mixture has been diluted to some convenient volume and the copper sulfide filtered off, the total creatine is determined colorimetrically in one portion of the filtrate and the preformed creatinine in another. (Creatinine, unlike free creatine, is

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[20] For the purposes of this paper the expression "residual organic phosphorus" will be used to indicate the organic phosphorus not in the form of phosphocreatine.

carried down in the copper precipitate.) The difference between the two results represents the combined creatine. Since phosphocreatine is partly hydrolyzed during the treatment with H_2S the labile phosphorus must be determined on a separate sample of the muscle filtrate.

Precipitation by copper, followed by extraction of the precipitate with alkali, is therefore capable of yielding a solution in which 93 per cent of the phosphorus is in the form of phosphocreatine. No other way has been found by which so much can be accomplished in a single operation, but unfortunately the copper method is less effective on a large scale, at least with ordinary laboratory apparatus. The manipulation of the bulky precipitate obtained from several hundred gm. of muscle consumes a considerable amount of time, during which some phosphocreatine is decomposed.[18] Using a shaking machine for the extraction we have been able to recover by this method about 60 per cent of the phosphocreatine present in the original muscle filtrate, the ratio of phosphocreatine P to total P in the extracts averaging 88 per cent.

Since experiments along other lines have meanwhile shown more promising results the copper method has been abandoned, temporarily at least. The preliminary separation according to this principle might nevertheless be conducted with less loss of material if the time required for extracting the precipitate could be shortened by means of special apparatus, in which case the use of copper followed by calcium might be an improvement on our present method. The usefulness of calcium, particularly for the removal of the last traces of residual organic phosphorus, had not yet been recognized while our experiments with the copper separation were in progress. The information that we have to offer on the further purification of the copper extract is consequently limited to methods, depending on the use of lead and barium, in which the yields are far from satisfactory.

Purification of Copper Extract.

Lead.—From an extract of the copper precipitate which has been concentrated[21] until it contains about 0.1 mg. of phosphorus

[21] Slightly alkaline solutions containing phosphocreatine can be concentrated under diminished pressure (at a temperature of about 40°) without decomposition.

per cc. and made slightly acid (pH 6) with acetic acid, lead acetate (0.2 volume of a 10 per cent solution) will precipitate about 30 per cent of the phosphocreatine, virtually free from organic impurities containing phosphorus.   Since the lead salt is unstable[18] the final yield is very poor, and this method has not been carried out except on a small scale.

Barium.—A considerable part of the residual organic phosphorus remaining in the copper extract can be removed by taking advantage of the comparatively slight solubility of phosphocreatine in solutions of barium hydroxide.   If a preparation containing 30 mg. of labile phosphorus is concentrated *in vacuo* to 20 cc. and treated with an equal volume of saturated barium hydroxide solution, about half the phosphocreatine will be thrown down in a fairly pure condition.   If the filtrate from this fraction is precipitated with alcohol, and the alcohol precipitate extracted with 30 cc. of saturated barium hydroxide solution in two or three installments, more phosphocreatine may be recovered from the residue left undissolved.   By repeating the entire process the ratio of phosphocreatine P to total P can be increased to about 95 per cent, but the further purification of this material is more difficult to carry out.   Precipitation with alcohol from hot solution gives a product which is partly crystalline, but this procedure has not been uniformly successful in our hands unless the impurities have been substantially all removed before the hot precipitation is attempted.

By the following method, which is entirely empirical and the success of which we believe to depend upon the removal of residual organic phosphorus by adsorption, it is possible to get crystalline material in which no amorphous particles can be detected with the microscope.

In this case the alkaline extract of the copper precipitate (containing about 0.3 mg. of phosphorus per cc.) is treated with a freshly prepared solution of sodium sulfide (in sufficient excess to cause agglutination of the copper sulfide formed), and the mixture filtered.   A saturated solution of barium hydroxide is then added to the filtrate, a little more than enough to cause complete precipitation being used.   The precipitate (barium carbonate, *etc.*) is filtered off and the phosphocreatine recovered from the filtrate by adding alcohol (2 volumes).   The atomic ratio of

barium to phosphorus at this stage is well over 1:1, and before crystallization is attempted the excess barium must be removed. This may be accomplished by adding about 5 cc. of water for each mg. of phosphorus present and leaving the mixture exposed to the air in an open dish until it is no longer alkaline to phenolphthalein. If the barium carbonate which has now formed is filtered off, and the clear filtrate heated to the boiling point, the phosphocreatine can be precipitated as the secondary salt (crystallizing in spherulites) by the gradual addition of 2 volumes of alcohol to the gently boiling mixture. This process cannot be · safely carried out except on a relatively small scale. The volume of the aqueous solution must be small enough so that the boiling point is reached within a few seconds, otherwise there is danger of hydrolysis. On the other hand, by working with a small volume of solution at one time hydrolysis can be avoided, and the substance even crystallized a second time in the same way without detectable decomposition. After the second crystallization the labile phosphorus and the total phosphorus should be identical.

As stated in the introduction, methods in which barium is used give excessively low yields (not more than 5 per cent). Consequently, besides being of little value as methods for preparing phosphocreatine, they fall far short of furnishing acceptable evidence that creatine and phosphoric acid are the sole constituents of the natural product. Various other procedures depending on some sort of fractionation of the barium salt for the last · steps of the isolation have been tried, without material improvement in the yield.

In some of these preparations the preliminary treatment with copper has been replaced by the following method, which gives about the same results. The inorganic phosphate, *etc.*, is removed from the neutralized trichloroacetic acid filtrate by the addition of barium hydroxide. The filtrate from the barium precipitate is adjusted to pH 7 with acetic acid and treated with 40 cc. of 10 per cent lead acetate per liter, which throws down a large proportion of the residual organic phosphorus. On account of the dilution and of the relatively small amount of lead the phosphocreatine remains dissolved, and may be recovered from the filtrate by adding alcohol (1.5 volumes). In order to remove the lead without the loss of a large amount of material by hydrolysis

the temperature must be kept low.   The alcoholic lead precipitate is separated by filtration as rapidly as possible, dissolved at once in ice water, and the lead precipitated as the sulfide.   During all these operations, and also during the subsequent removal of the excess H_2S by aeration, cooling in a bath of ice water is essential. As soon as all the H_2S has been expelled the solution is neutralized with barium hydroxide and the lead sulfide filtered off.   (Before the neutralization with barium hydroxide the lead sulfide is more or less colloidal.)   The solution of the crude barium salt may now be concentrated by precipitating with alcohol and redissolving the precipitate in water.   In case it is desired to obtain the substance in some other form the barium should not be removed with sulfate, for barium sulfate adsorbs phosphocreatine.   The barium salt may however be suspended in a small amount of water and triturated with a solution of sodium or potassium carbonate.   Barium-free solutions containing as much as 2 or 3 mg. of labile phosphorus per cc. can in this way be prepared with very little loss.

In addition, a number of other metals have been used in place of barium; *e.g.*, strontium, magnesium, lead, zinc, and silver, with or without the addition of alcohol to decrease the solubility.   In a general way our experience with all these reagents has been the same.   The separation can be carried satisfactorily to a certain point, but eventually further reprecipitations, whether fractional or not, either fail to change the composition or at best give a slightly purer product in distressingly poor yield.   Even repeated precipitation of the calcium salt with alcohol, which forms the basis of the method given in the section following, may be ineffective as long as the phosphocreatine comes down in amorphous form, but once a portion of it has been induced to crystallize each succeeding reprecipitation increases the ratio of phosphocreatine P to total P until finally the last trace of impurity has been removed.

Isolation of Phosphocreatine from Muscle as the Calcium Salt.

Preparation of Protein-Free Filtrate.—All our experiments on the isolation of phosphocreatine have been made with muscle from cats anesthetized with amytal.   The muscle masses are cut away as rapidly as possible, at the same time avoiding all unnecessary stimulation, and at once *immersed* in liquid air.   As each mass

becomes frozen through, it is transferred to an iron mortar, kept frozen by adding small amounts of liquid air at intervals, and ground with an iron pestle.   Altogether about 500 cc. of liquid air should be used for each 100 gm. of muscle.   The ground and still frozen muscle is weighed as rapidly as possible and immediately placed in a large porcelain mortar containing ice-cold 5 per cent trichloroacetic acid (1 liter for each 100 gm. of muscle). The mixture is now thoroughly stirred up, and the sediment allowed to settle out.   The supernatant fluid is poured off into a large flask, and the residue passed through a meat chopper and then returned to the trichloroacetic acid solution, where it should be left for an hour or two, with occasional shaking to facilitate extraction of the phosphocreatine.[22]   After standing for 2 hours at the most the suspension is filtered,[23] and the filtrate promptly neutralized to phenolphthalein with a saturated solution of c.p. sodium hydroxide which has been made up in a dish of metal not readily attacked by alkali.[24]   *All operations up to this point should be conducted in a cold room kept at a temperature of 0° or lower.*   The effect of hydrolysis of phosphocreatine by enzyme action or by acid is not merely to decrease the yield; an even more serious result is the consequent increase in the proportion of impurities, entailing a larger number of recrystallizations in order to get the product pure.

Removal of Inorganic Phosphate, Etc.—To each liter of the neutralized trichloroacetic acid filtrate are added 100 cc. of a 10 per cent solution of calcium chloride ($CaCl_2$) saturated with calcium hydroxide.   The precipitate is allowed to settle, the supernatant fluid siphoned off, and the sediment filtered.   The combined filtrate and supernatant fluid, which will be designated as the

[22] During this time a small amount is hydrolyzed, but the recovery is nevertheless increased because of more complete extraction.   The statement recently made by Irving and Wells that no loss of labile phosphorus occurs in 5 hours at 0° is not correct (Irving, L., and Wells, P. H., *J. Biol. Chem.*, **77**, 97 (1928).

[23] If it is possible to arrange for suction filtration in the cold room the yield of filtrate can be increased by about 10 per cent.

[24] The silicate present in alkali solutions which have been prepared and kept in glass seriously affects the yield by interfering with the extraction of the precipitated calcium salt with water.   Iron has a similar effect, hence the necessity of using sodium hydroxide of good quality.

"calcium filtrate," should amount to about 975 cc. for each 100 gm. of muscle used, and if proper care has been exercised in the preparation should contain approximately 35 mg. of labile phosphorus per liter.

Precipitation with Alcohol.—In this first precipitation of the phosphocreatine the temperature again becomes important. In order to get approximately complete precipitation (about 95 per cent), 3 volumes of alcohol must be added and the mixture let stand overnight. Meanwhile, unless special precautions are taken to avoid it extensive hydrolysis will occur. The amount of phosphocreatine hydrolyzed at this stage may be cut down to less than 10 per cent (1) by using ice-cold alcohol and (2) by neutralizing[24] the alcohol (to phenolphthalein) before it is added to the calcium filtrate and keeping the mixture slightly alkaline during the precipitation, which of course must take place in the cold room. Owing largely at least to impurities in commercial alcohol the mixture tends to become acid on standing, even in the cold. The addition of alkali during the precipitation may be omitted if the alcohol used has been distilled from lime.

The use of more than 3 volumes of alcohol, for the sake of recovering the additional 5 per cent of phosphocreatine, is inadvisable. Under the prescribed conditions about one-third of the residual organic phosphorus remains dissolved. If the alcohol concentration exceeds 75 per cent, more of this impurity comes down, and its subsequent removal complicates the later stages of the isolation so that the final yield of phosphocreatine is actually less.

The alcoholic calcium precipitate, which is already partly crystalline, is separated by centrifugation on the next day, and without being allowed to dry[25] is extracted with water according to the following directions.

Extraction of Precipitate.—In the preceding step the more

[25] At each stage of the preparation the precipitated calcium salt should be redissolved in water as soon as it has been filtered off. The salt dissolves in water much more rapidly if it is still moist, but the chief objection to delay is that the hydrolysis of the calcium salt of phosphocreatine in concentrated aqueous solution, or in the form of a partially dried solid, is an autocatalytic process. When the conditions are such that the calcium *o*-phosphate formed precipitates, it carries down excess of base, leaving the aqueous phase more acid than before.

soluble impurities have been to a large extent eliminated at the expense of only a small amount of phosphocreatine. By again sacrificing about 5 per cent of the desired substance a considerable amount of relatively insoluble material containing phosphorus can now be gotten rid of. If the precipitate is extracted with only a limited amount of water another third of the residual organic phosphorus remains behind in the residue, and the ratio of phos phocreatine P to total P in the extract will be approximately 85 per cent. When this degree of purity has been attained the remainder of the process is merely a matter of a few reprecipitations of the calcium salt with alcohol.

The water used for the extraction of the alcoholic calcium precipitate is about equal in amount to the weight of muscle taken, and is divided into four installments. For example, if the preparation was begun with 400 gm. of muscle (a convenient quantity, capable of yielding approximately 1 gm. of the pure calcium salt), the precipitate is extracted with 400 cc. of water, 100 cc. at a time. After each individual extraction the mixture may be either filtered with suction or centrifuged, according to convenience. The residue is then extracted with a second 100 cc. of water, and so on.

Further Purification.—The four extracts are now combined into one solution, and the rest of the impurities removed by repeated precipitation with 1 volume of alcohol.[26] Not more than four reprecipitations should be necessary if the directions given below are closely followed. Here it is worth while to use alcohol which

[26] The time required for the preparation may be shortened, at the risk however of losing the entire batch, by crystallizing the calcium salt at this point from hot solution; *i.e.*, by the gradual addition of 0.5 volume of alcohol to the extract, which has first been made distinctly alkaline with saturated calcium hydroxide solution (0.1 cc. per mg. of phosphorus) and heated rapidly to the boiling point. If the mixture is kept gently boiling for a minute or two after all the alcohol is added the substance may crystallize out promptly (in the form of needles, generally arranged in rosettes). On the other hand, in some preparations where this method has been tried the formation of crystals for some reason was delayed, and when this happens most of the phosphocreatine is decomposed. The method of hot precipitation either works very well, giving at once a product containing little or no residual organic phosphorus, or it does not work at all. At best the yield is distinctly less than by the slower method in which no heat is used.

has been refluxed and distilled over lime; whether it is absolute or not is immaterial. For the first two reprecipitations the procedure is as follows:

To the combined aqueous extracts, containing 0.25 to 0.3 mg. of labile phosphorus per cc., is added 0.5 volume of purified alcohol. Some amorphous material will come down at once, especially in the first precipitation. The mixture is now stirred at frequent intervals and the wall of the containing vessel scratched with a glass rod. After half an hour or more crystals (spherulites) begin to appear. On further stirring these increase in size and quantity, and the amorphous material precipitated earlier also becomes largely crystalline. About 3 hours later the mixture is again stirred thoroughly and a second 0.5 volume of alcohol run in. On the next day the precipitate is filtered off with suction (washing is unnecessary) and redissolved in water enough to make the concentration of labile phosphorus again between 0.25 and 0.3 mg. per cc. Unless the solution is now alkaline to phenolphthalein it should be made so with a saturated solution of calcium hydroxide.

The second reprecipitation is conducted in precisely the same way. This time much less amorphous material appears, and crystallization begins sooner (within 5 minutes if the solution is well stirred).

The ratio of phosphocreatine P to total P should now be about 95 per cent, and that is about as far as the purification can be carried without making a slight change in the method of precipitation. In order to eliminate the last 5 per cent of residual organic phosphorus the substance must be made to separate in the form of larger crystals. In other words the crystallization has to be retarded, and not aided as before. To prevent the immediate appearance of crystals in the third reprecipitation, therefore, the solution must be filtered before the alcohol is added. The filtration serves to remove any traces of crystalline material which might seed the mixture, and at the same time incidentally disposes of the small amount of amorphous calcium phosphate which has accumulated in the preparation as a result of phosphocreatine hydrolysis. The alcohol is added as usual, 0.5 volume at a time,— not omitting first to make the solution alkaline to phenolphthalein if necessary,—but now except for the few strokes of the glass rod required to insure thorough mixing the stirring is dispensed with.

Even without any mechanical assistance crystallization begins in the course of a few minutes. The second 0.5 volume of alcohol may be added after an interval of 3 hours, and the mixture filtered the next day, exactly as in the first two reprecipitations. After the fourth reprecipitation, which is exactly like the third, the labile phosphorus and the total phosphorus should be identical within the error of analysis. The final product is at once dried over sulfuric acid *in vacuo*.[25] In Table I analytical data are given showing the progress of the isolation in a typical experiment.

Although all detectable traces of organic impurities containing phosphorus are removed in the process described above, the

TABLE I.
Purification of Phosphocreatine.

	Phospho-creatine P.	Residual organic P.	Phosphocreatine P. / Total P
	mg. per 100 gm. muscle	*mg. per 100 gm. muscle*	*per cent*
Original calcium filtrate....................	40.3	20.0	67
Precipitated with 3 volumes alcohol.........	35.0	12.8	73
Aqueous extract of above precipitate........	32.9	6.4	84
1st reprecipitation (1 volume alcohol).......	30.6	3.5	90
2nd " (1 " ").......	29.4	1.8	94
3rd " (1 " ").......	28.2	0.5	98
4th " (1 " ").......	27.5	0	100

product is still strictly speaking not a single substance. Since the various reprecipitations have been conducted from alkaline solution—to avoid hydrolysis as far as possible—the ratio of calcium to phosphorus is higher than the secondary salt of phosphocreatine requires. Furthermore, the composition is not what it theoretically should be even on a calcium-free basis, for the slightly alkaline solutions absorb carbon dioxide from the air and hence some carbonate is carried down with each alcohol precipitation. A little inorganic phosphate is likewise present. The analysis of one such preparation follows.

The substance was dried *in vacuo* over sulfuric acid and then allowed to come to constant weight in air.

Analysis.—$C_4H_8O_5N_3PCa \cdot 4H_2O$.

Calculated.   " 14 94, H 5.01, N 13 08, P 9.66, Ca 12.47, creatine 40.8.
Found.   " 15.08, "4.71, " 12.78, "9.49, " 14.49,   "   39.3.

In this and subsequent analyses nitrogen was determined by the Kjeldahl method; creatine colorimetrically; phosphorus (after ashing with sulfuric and nitric acids) by titration of the magnesium ammonium phosphate precipitate;[27] and calcium by ignition of the oxalate precipitate followed by alkalimetric titration of the calcium oxide.

While the substance so prepared contains no detectable extraneous organic matter,[28] and should hence be pure enough for many purposes, for such matters as the construction of a titration curve it will not suffice.   In the next section will be found a method for making the pure secondary salt, free from carbonate and inorganic phosphate.

Preparation of Secondary Calcium Salt.

The calcium : phosphorus ratio in preparations made according to the above directions is subject to considerable variation.   We have found it as low as 1.06 atoms of calcium to 1 of phosphorus and as high as 1.16.   The removal of the excess calcium, as well as of both carbonate and inorganic phosphate, may be accomplished at one time in the following way.

A solution of the substance containing about 0.4 mg. of phosphorus per cc. is first made alkaline with an equal volume of saturated calcium hydroxide, and the precipitate of calcium phosphate and carbonate filtered off.   The filtrate is cooled to 0° with the least possible exposure to the air, then made distinctly acid (to brom-cresol purple) with dilute hydrochloric acid, again filtered rapidly in case any undissolved calcium carbonate remains, and at once precipitated by the gradual addition of 3 volumes of ice-cold alcohol.   During the addition of the alcohol the mixture should be stirred continuously.   After 30 minutes standing in the

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[27] Fiske, C. H., *J. Biol. Chem.*, **46**, 285 (1921).

[28] 9.41 mg. of the above preparation, dissolved in acid, liberated 0.080 mg. of carbon dioxide, or 0.85 per cent, equivalent to 0.23 per cent of carbon. When this is subtracted from the 15.08 per cent of carbon found by combustion the result is 14.85 per cent, making the corrected atomic ratio of carbon to nitrogen 4.07:3, or very nearly the theoretical ratio for phosphocreatine.

cold room the precipitate is filtered off with suction, washed with alcohol, and dried as rapidly as possible over sulfuric acid in a vacuum desiccator.[25]   Since the desiccated salt is very hygroscopic, all analyses have been made with material which has come to constant weight in air after being dried *in vacuo*.[29]

Analysis.—$C_4H_8O_5N_3PCa.4H_2O$.
Calculated.   C 14.94, H 5.01, N 13.08, P 9.66, Ca 12.47, creatine 40.8,
H₂O (3½ molecules) 19.6.
Found.   C 14.98, H 5.11, N 13.04, P 9.75, Ca 12.62, creatine 40.2,
H₂O 20.0.[30]

Isolation of Creatine after Hydrolysis.

In the course of some earlier attempts to purify phosphocreatine as the free acid, which owing to the instability of the substance met with no success, we had many opportunities to observe the formation of crystals identical in form with those of creatine.   Beyond the fact that it responded to the Jaffé test after heating with hydrochloric acid, no further characterization of this product of hydrolysis was undertaken until pure phosphocreatine had been prepared.   Even now this is a point of relatively slight importance, for Baumann and Ingvaldsen[31] showed some time ago that the creatine content of muscle extracts is nearly the same whether determined by the usual colorimetric methods or by isolation as creatinine picrate; or in other words that as far as the Jaffé reaction is concerned 90 per cent or more of the chromogenic substance present is creatine.   For the sake of completeness, however, the following results are given.

100 mg. of the pure calcium salt of phosphocreatine were dissolved in 20 cc. of water, and hydrolyzed by heating for 2 minutes in a boiling water bath.   The mixture was made definitely

[29] The water content varies slightly with the humidity.

[30] At a pressure of 0.1 mm. of mercury over phosphorus pentoxide most of the water is driven off at 20°.   At higher temperatures very little more is ·lost, and between 80° and 110° there is no change.   Even at the latter temperature about 0.5 molecule of water appears to be retained, and this we have not been able to remove without signs of incipient decomposition. The behavior of the salt when heated depends upon the water content at the start.   Unless it is first well dried at room temperature *in vacuo* some destruction will occur even at the boiling point of ethyl alcohol (78°).

[31] Baumann, L., and Ingvaldsen, T., *J. Biol. Chem.*, **25**, 195 (1916).

alkaline (pH 9) with a solution of calcium hydroxide and centrifuged to remove the precipitated calcium phosphate, which was washed with a few cc. of warm water. The clear supernatant fluids were combined, exactly neutralized with hydrochloric acid, and evaporated to dryness *in vacuo* over sulfuric acid. To the residue, dissolved in 2 cc. of warm water, were added 20 cc. of hot absolute alcohol. The mixture was allowed to cool to room temperature, and finally let stand overnight at 0°. The crystals were then filtered off, and weighed 42.8 mg. The solubility correction, based on control experiments with pure creatine, was found to be 2.4 mg., making the corrected yield 45.2 mg. The theoretical yield of creatine hydrate is 46.4 mg.

Analysis.—$C_4H_9O_2N_3 \cdot H_2O$.
Calculated. N 28.19, H_2O 12.08, creatine 87.9.
Found.   " 28.17, " 12.13,   "   88.6.

Structure and Properties of Phosphocreatine.

The above analyses of the calcium salt indicate that we are dealing with a substance which, in the form of the free acid, has the composition $C_4H_{10}O_5N_3P$, and contains 1 molecule of creatine and 1 of *o*-phosphoric acid. The composition of the secondary calcium salt is not changed by two more reprecipitations with alcohol provided that hydrolysis is prevented by observing the precautions mentioned. So far no other stable salt which crystallyzes readily and which can be obtained in a form suitable for analysis has been found, and none of the amorphous salts which we have been able to prepare gives consistently satisfactory analyses.

Precipitation with alcohol in the presence of a large excess of calcium hydroxide gives needles (mostly arranged in burrs), which presumably represent the tertiary calcium salt, but this product is attacked by atmospheric CO_2. As stated earlier, crystallization of the secondary barium salt by adding alcohol to the hot aqueous solution results in some hydrolysis except when conducted on a very small scale. When alcohol is added at room temperature to an aqueous solution of the barium salt (prepared from the *pure* calcium salt) an amorphous precipitate is formed. On long standing this gradually becomes crystalline, but meanwhile it has been

partly decomposed. Finally, mercuric acetate gives a micro-crystalline precipitate which does not keep.

Among the amorphous precipitates which can be obtained from concentrated aqueous solutions may be mentioned the salts of calcium, barium, strontium, and lead. In addition, phospho-creatine is precipitated even from comparatively dilute solutions by silver nitrate and barium hydroxide, behaving in this respect like creatine itself; the precipitate is colorless at first, but soon darkens when exposed to light.

With silver nitrate alone, in neutral or slightly acid solution, no precipitate appears, but a silver salt can be obtained by the addition of alcohol or acetone. This salt, although not particularly sensitive to light, soon undergoes hydrolysis even in the dry state. Alcohol will also precipitate the lead and magnesium salts from dilute aqueous solution. All these products are amorphous.

Accepting the structure commonly assigned to creatine, we may suppose the constitution of phosphocreatine to be as follows:

$$HN : C \overset{\displaystyle NH \cdot PO(OH)_2}{\underset{\displaystyle N(CH_3) \cdot CH_2 \cdot COOH}{\Big\langle}}$$
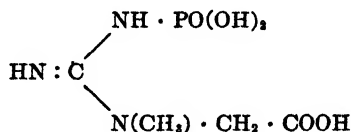
As long as there is any doubt about the constitution of creatine itself it is hardly worth while attempting to decide which nitrogen atom is combined with the phosphoric acid group. The only organic compounds of this type which have been prepared before are phosphoric acid derivatives of comparatively simple primary amines. Michaelis[32] has synthesized a number of acid halides of the type $R \cdot NH \cdot POCl_2$ (and $R \cdot NH \cdot POBr_2$), but with the exception of a few relatively insoluble aromatic representatives the corresponding acids $(R \cdot NH \cdot PO(OH)_2)$ were found to be so unstable that they could not be isolated. The free phosphamic acids which Michaelis was able to prepare were obtained from the chloride or bromide by removing the halogen in alkaline solution (with or without the use of heat), followed by precipitation with

[32] Michaelis, A., and Luxembourg, K., *Ber. chem. Ges.*, **29**, 710 (1896). Michaelis, A., *Ann. Chem.*, **326**, 129 (1903).

hydrochloric acid, and even these were unstable in solution. Phosphocreatine resembles these synthetic products in being stable in alkaline solution and readily hydrolyzed by acid.

Acid Hydrolysis.—While the velocity of acid hydrolysis of esters, sucrose, *etc.*, is proportional, or at any rate approximately proportional, to the hydrogen ion concentration (or activity),[33] no such relationship holds in the present case except perhaps over a very

TABLE II.
Acid Hydrolysis of Phosphocreatine (22°).

HCl concentration.	pH	Buffer.	Time.	Per cent hydrolyzed.	k*	Mol fraction of primary salt (p).	$\dfrac{k}{C_H \cdot 10^3}$	$\dfrac{k}{p}$
N			*hrs.*					
0.5			1	31.3	0.163		0.0004	
0.1			1	29.7	0.153		0.002	
	3.0	Phthalate†	1	25.9	0.130	0.97	0.13	0.134
	3.4	" †	1	25.2	0.126	0.93	0.32	0.136
	3.8	" †	1	22.7	0.112	0.84	0.70	0.133
	4.2	" ‡	1	21.5	0.105	0.68	1.66	0.154
	4.6	Acetate§	2	27.2	0.069	0.46	2.76	0.150
	5.0	" §	2	13.3	0.036	0.25	3.60	0.144
	5.4	" §	2	7.2	0.0162	0.12	4.07	0.135
	5.8	" ‖	5	6.5	0.0058	0.05	3.65	0.116
	6.2	" ‖	5	1.7	0.0015	0.02	2.38	0.075

* $\dfrac{1}{t} \log \dfrac{a}{a-x}$.

† Brom-phenol blue.
‡ Brom-cresol green.
§ Methyl red.
‖ Brom-cresol purple.

limited range. On passing from alkaline solution—where phosphocreatine remains intact for days—through the neutral point to the acid side there is a gradual increase in the hydrolysis velocity, more or less closely following the rise in C_H. Above pH 5.4 the data that we have (Table II) are very rough because the

[33] See *e.g.* Terry, E. M., *J. Am. Chem. Soc.*, **50**, 1239 (1928). Rice, F. O., The mechanism of homogeneous organic reactions from the physical-chemical standpoint, New York, 1928, 109–127.

amount of inorganic phosphate formed under the conditions used is too small to measure accurately, but as the acidity increases from this point the ratio of the velocity constant to the hydrogen ion concentration $\left(\dfrac{k}{C_H}\right)$ progressively diminishes, in fact beyond pH 3.4 further increase in the acidity has comparatively little effect on the rate of the reaction. At pH 1.1 (*i.e.* in 0.1 N hydrochloric acid) the rate is only about 20 per cent higher than at

TABLE III.

Titration of Secondary Calcium Salt of Phosphocreatine (1 Mol) with Hydrochloric Acid.

HCl added.	pH		Indicator.	pK'$_1$
	Colorimetric.	Electrometric (24°).		
mols				
0.04	5.8		Methyl red.	(4.42)
0 07	5 6		" "	(4.45)
0.13	5.4		" "	4.57
0 20	5.2		" "	4.60
0 28	5.0		" "	4.59
0.37	4.8		" "	4.57
0 49	4.6		" "	4.58
0 50		4.55		
0 62	4.4		Brom-phenol blue.	
0.73	4.2		" "	
0.87	4.0		" "	
0.98	3.8		" "	
1.00		3.74		

pH 3.4, and the effect of increasing the acidity from 0.1 to 0.5 N is slight.

Within the range where the solution contains a mixture of the primary and secondary salts of phosphocreatine—that is, between neutrality and the neighborhood of pH 3—there is on the other hand a well defined relationship between the rate of the reaction and the composition of the mixture. The velocity constant in this range is proportional to the mol fraction of the primary salt, as may be seen from the last column of Table II. For the data on which these calculations have been based it will be necessary

to refer to the titration curve, which is discussed below, and to Table III.[34]

It appears therefore that the primary salt of phosphocreatine is unstable in solution, whereas the secondary salt is not. A similar case has been observed in the conversion of the cinchona alkaloids to their respective toxines.[35] In mixtures of the primary and secondary salts of cinchonine, for instance, the rate of the reaction is proportional to the mol fraction of the former.

The measurements of hydrolysis velocity were made as follows. 1 cc. of a solution of the calcium salt of phosphocreatine (containing 0.31 mg. of phosphorus) was measured into a centrifuge tube. In the first two experiments of Table II, hydrochloric acid of the proper strength was added to bring the acidity to the desired point and to make the total volume 4 cc. In all other cases the acidity was first adjusted roughly with a trace of hydrochloric acid, after which 3 cc. of a 0.05 M buffer mixture (phthalate or acetate) of the required pH were added. (This amount of buffer is sufficient to maintain the pH constant during the reaction within the limits of the indicator method.) ·

The inorganic phosphate formed in each experiment was determined by the calcium precipitation method described later in this paper (p. 665).

Titration Curve.—Owing to the rapidity with which phosphocreatine is hydrolyzed in acid solution—a change which, as will be seen, is accompanied by a shift in pH in the direction of diminishing acidity—accurate measurements cannot be made with the

[34] The values of $\frac{k}{p}$ are only approximate, chiefly for the reason that the basis for calculating the mol fraction (p) is more or less uncertain. The second dissociation constant has been determined by the titration of 0.005 M solutions of the secondary calcium salt (Table III). The hydrolysis experiments on the other hand were made with solutions which because of the buffer added had a considerably higher, and somewhat variable, ionic strength. For the purpose of these calculations we have for the sake of simplicity taken μ to be 0.04 (an average figure), and the apparent dissociation constant at this ionic strength is estimated to be 3.0×10^{-5}. The high values for $\frac{k}{p}$ in the middle range are at least in part explained by the relatively low ionic strength of the 0.05 M acetate buffers in the neighborhood of pH 5.

[35] Biddle, H. C., *J. Am. Chem. Soc.*, **37**, 2088 (1915).

hydrogen electrode.   The potential drifts upward at a rate which may be as high as 0.2 or 0.3 millivolts per minute.   By taking a number of successive readings and extrapolating back, under the assumption that the initial change is linear during the few minutes between the preparation of the solution and the first potentiometer reading, an approximation to the correct value can be obtained.   As a matter of fact, of course, the electrode and the solution never come to complete equilibrium, and the result may accordingly be subject to an error of a few hundredths of a pH unit.   The electrometric method has consequently been used only as an additional check on two of the more important points of the titration curve.   Otherwise we have relied on the indicator method, avoiding salt errors as far as that is possible by taking care to have the standards[36] and the phosphocreatine solution of about the same ionic strength.

Inasmuch as no crystalline salt of phosphocreatine with a univalent base has yet been made, dilute solutions (0.005 M) of the secondary calcium salt were titrated directly (with 0.02 N HCl), a procedure which involves much less uncertainty than the titration of solutions of the sodium or potassium salt prepared by treatment of the calcium salt with oxalate.   From the results (Table III) it will be seen that the most probable figure for the second acid dissociation constant at the concentration used is 2.6×10^{-5} ($pK'_2 = 4.58$).   On the acid side of pH 4.6 the first hydrogen atom becomes effective, and the titration data in this region cannot be used for the calculation of pK'_2.   The information to be obtained by carrying the titration beyond 1 mol of acid is of little value, for measurements of a higher degree of accuracy than is now attainable would be required to determine the individual constants in a region where both acid and basic dissociation are involved.   From the physiological standpoint only pK_2 is of importance, for there is no constant in the neighborhood of 10^{-7}.[37]

[36] The standards (acetate mixtures) were checked with the hydrogen electrode (Clark electrode vessel, saturated calomel electrode, Leeds and Northrup Type K potentiometer).

[37] E.g. only 0 06 mol of NaOH (per mol of phosphocreatine) is required to titrate to pH 9.3.

According to the prevailing view[38] free creatine in aqueous solution would exist largely in the form of an internal salt.

$$HN : C \Big\langle \begin{matrix} NH_2{}^+ \\[1em] N(CH_3) \cdot CH_2 \cdot COO^- \end{matrix}$$
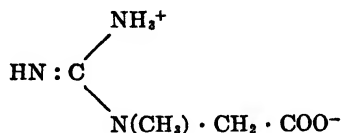
The presence in phosphocreatine of an acid group about equal in strength to that of the lower fatty acids indicates that here there

TABLE IV.

Base Liberated by Hydrolysis of 1 Mol of Phosphocreatine (0.005 M) at Constant pH.

pK$'_2$ of phosphocreatine 4.6.*
pK$'_2$ of o-phosphoric acid 7.0.

pH	Base liberated.
	mols
7.5	0.24
7.0	0.50
6.5	0.75
6.0	0.87
5.8	0.88†
5.5	0.86
5.0	0.70

* The true pK$_2$ of phosphocreatine (*i.e.* at infinite dilution), assuming that the relative activities of the primary and secondary salts are about the same as for the corresponding salts of o-phosphoric acid, is approximately 4.7.

† Maximum.

is no internal salt formation, or in other words that the carboxyl group is free. Regardless of any theoretical considerations, however, the hydrolysis of phosphocreatine in mixtures of its primary and secondary salts actually does result in the liberation of a considerable quantity of base, as shown in Table IV. For example, under optimum conditions (pH 5.8) the hydrolysis of 1 mol of phosphocreatine sets free 0.88 mol of base.[39] That is to

[38] See Bjerrum, N., *Z. physik. Chem.*, **104**, 52 (1923).

[39] By way of direct confirmation of this figure the following experiment may be cited. 4 cc. of a solution of the secondary calcium salt containing

say, at pH 5.8 the simultaneous hydrolysis of 1 mol of phospho-creatine and formation of 0.88 mol of lactic acid from a neutral substance (*e.g.* glycogen) would cause no change in hydrogen ion concentration. The following equation is an approximate representation of the reaction as it would occur (at pH 5.8) in a solution in which lactic acid is being formed from glycogen.

$$0.94 \ C_4H_8O_5N_3PK_2 + 0.06 \ C_4H_9O_5N_3PK + 0.445 \ C_6H_{10}O_5 + 1.445 \ H_2O =$$
$$0\ 06 \ K_2HPO_4 + 0.94 \ KH_2PO_4 + C_4H_9O_2N_3 + 0.88 \ C_3H_5O_3K + 0.01 \ C_3H_6O_3$$
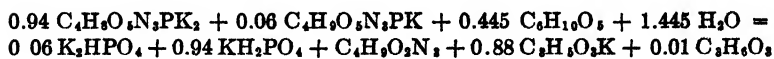
The pH at which the maximum amount of base is liberated naturally varies with the electrolyte concentration (ionic strength) of the medium. The data of Table IV are based on the titration curve of a 0.005 M solution of the secondary calcium salt (Table III). At the ionic strength of muscle (presumably in the vicinity of 0.15) the optimum pH would be shifted towards the acid side, roughly to pH 5.6.

The extent to which this neutralizing mechanism[11] may be supposed to operate in working muscle depends of course upon the pH of the cell interior. While many measurements have been made on muscle extracts, the only technique yet devised which shows the pH within the muscle fibers as distinguished from the fluid bathing them is the method of intravital staining used by Rous and his collaborators. Rous[40] was able to show that the contents of the intercellular spaces of mammalian voluntary muscle are definitely alkaline; the fibers on the contrary assume the acid shade with phenol red, brom-cresol purple, and even chlor-phenol red.[41] From these results, as Rous concludes, the muscle cell must be at least as acid as pH 5.6. Gross errors, such as might be caused by the effect of protein and other substances

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0.727 mg. of phosphorus were adjusted to pH 5.9 with hydrochloric acid. 1.34 cc. of 0.02 N HCl were then added, and the solution was heated to boiling, cooled, and titrated back with 0.02 N NaOH. The volume of alkali required was 0.33 cc. Consequently 1.34 − 0 33 = 1 01 cc. of 0 02 N base were liberated during the hydrolysis, or 0 86 mol of base per mol of phosphocreatine.

[40] Rous, P., *J. Exp. Med.*, **41**, 379, 399, 451, 739 (1925).

[41] Acidosis incident to the use of ether does not account for these results. We have been able to confirm them using animals (guinea pigs and mice) anesthetized with amytal.

on the color of the indicator, have been excluded by numerous control experiments.[42] While the accuracy which can be obtained with indicators in the case of clear solutions is hardly possible in a method of this kind, there is no obvious reason to doubt that Rous' results approximate the truth.

In muscle of average composition (0.45 per cent phosphocreatine) the hydrolysis of all the phosphocreatine would suffice, if the contents of the fiber are as acid as Rous' observations indicate, to neutralize 0.17 per cent of lactic acid (0.88 equivalent), the pH remaining constant. Since the resting lactic acid content of mammalian muscle averages about 0.06 per cent,[43] no change in the acidity would occur under these conditions until the amount of lactic acid present reached 0.17 + 0.06, or 0.23 per cent,[11] unless of course there is still some important reaction, not yet discovered, which is capable of affecting the pH to a significant extent.

It is not our intention to suggest that the pH of the contracting muscle fiber is actually constant. As a matter of fact, although analysis of muscle extracts shows that complete fatigue is accompanied by an increase in the acidity,[44] nothing whatever is known about the situation within the muscle cell. Considering the by no means remote possibility that other chemical reactions involved in the acid-base equilibrium of muscle are yet to be disclosed, the value of calculations based—as they must be—only on the reactions which happen to be known at present is obviously limited. Unfortunately, if a procedure as insensitive as intravital staining is the only method of approach the prospect of direct measurement seems equally unpromising, particularly in the initial stages of contraction when any change in pH that might occur would probably be small.

Finding that muscular activity is accompanied by the production of a small amount of ammonia (from adenylic acid), and

[42] Drury, D. R., and Rous, P., *J. Exp. Med.*, **43**, 669, 687 (1926).

[43] For data on cat muscle taken from the living animal and frozen with liquid air see Simpson, W. W., and Macleod, J. J. R., *J. Physiol.*, **64**, 255 (1927). Most of their figures lie between 0.05 and 0.09 per cent. While there is little doubt that mechanical manipulation is responsible for a part of the lactic acid found in mammalian muscle which has not been intentionally stimulated, it is equally probable that the phosphocreatine content would be higher if all such manipulation could be dispensed with.

[44] Meyerhof, O., and Lohmann, K., *Biochem. Z.*, **168**, 128 (1926).

believing that lactic acid production does not take place until after contraction has occurred, Embden[45] has suggested that the pH *increases* at the moment of contraction. If this should be true the hydrolysis of phosphocreatine is more likely to be the major cause, for the amount of adenylic acid decomposed is relatively slight. In this connection it may be noted that, even before the existence of any reaction capable of liberating base during muscular contraction had been demonstrated, Tiegs[46] presented the hypothesis that acid is set free in one part of the cell and alkali in another.

Since current views on the mechanism of muscular contraction were developed at a time when the only major processes known to influence the pH of muscle were the production of lactic acid from glycogen (and the reverse reaction) on the one hand, and a few relatively ineffective buffer mechanisms on the other, it is to be expected that some realignment will now be required. Possible bearings of the new neutralizing device represented by phosphocreatine hydrolysis on the supposed rôle of lactic acid in the initiation of contraction, on the question of fatigue, *etc.*, have been mentioned in a previous communication.[11]

Determination of Phosphocreatine and Inorganic Phosphate in Voluntary Muscle.

Preparation of Protein-Free Filtrate.

The main precautions to be observed in preparing the protein-free filtrate are (1) to subject the muscle to the least possible handling when it is removed, and (2) as far as possible to avoid hydrolysis by acid during the precipitation of the protein. The danger of acid hydrolysis is slight if the temperature is not allowed to rise much above the freezing point and if contact with the acid is not unnecessarily prolonged. Enzyme action, on the other hand, cannot be avoided altogether if the muscle is under strictly physiological conditions just before it is cut out. Only a few of the smaller muscles have tendinous insertions at both ends, hence generally the tissue must be separated from the bone by cutting through the fibers at one end at least.

[45] Embden, G., *Ber. ges. Physiol. u. exp. Pharmakol.*, **38**, 157 (1927); *Klin. Woch.*, **6**, 628 (1927).

[46] Tiegs, O. W., *Australian J. Exp. Biol. and Med. Sc.*, **1**, 11 (1924-25).

In order to insure rapid penetration by the protein-precipitating agent the muscle sample should not weigh more than 2 or 3 gm. In case liquid air is used the frozen muscle may be broken up and a piece of proper size selected for analysis, otherwise a section (preferably of 3 gm. or less) has to be cut off while the muscle is still irritable.[47]   It will be seen readily therefore, that the results obtained for inorganic phosphate in the case of resting muscle are bound to be somewhat higher than they should be, and those for phosphocreatine correspondingly too low.

The method which we now use for routine comparative analyses when liquid air is not at hand is as follows: The entire muscle is cut away from its insertions as quickly as careful handling will permit.   This is done in close proximity to a cold room kept just below 0°.   The muscle is then carried promptly to the cold room, where it is weighed as rapidly as possible on a torsion balance (or 2 to 3 gm. cut off and weighed in the case of larger muscles). The subsequent manipulation is the same whether liquid air has been used or not.

The weighed sample is at once transferred to a chilled porcelain mortar containing 20 to 30 cc. of ice-cold 5 per cent trichloroacetic acid (*i.e.* enough to give an approximately 1:10 dilution).   After about a minute's standing (to cool the tissue) the liquid is poured off into a small flask, and the muscle residue *thoroughly* ground with about twice its weight of *cold* quartz sand, an operation which can be completed in about 5 minutes.[48]   The trichloroacetic acid solution in the flask is then returned to the mortar, and the whole well stirred with the pestle, the mixing being finally completed

[47] The corresponding muscles of the right and left sides do not, except occasionally, give the same results even when the two samples are prepared by the same method.   The inorganic phosphate is often 10 per cent higher on one side, either because the composition of the two muscles is not identical during life or because they do not react in precisely the same way when they are dissected out.   On this account the comparison of two different methods of preparation is not a simple matter, and we are unable to say positively that anything is gained by the use of liquid air as long as the muscle sample is not large.   It is quite possible that the contraction which occurs when the muscle comes into contact with the liquid air may be enough to balance the advantage of more rapid cooling.

[48] If the muscle is simply cut up with scissors, and not ground, extraction will not be complete for several hours, and in the meantime the phospho-creatine will be partly decomposed.[22]

by pouring the supernatant fluid once more into the flask and back again. Without further delay the mixture is now filtered, and the filtrate immediately made just alkaline to phenolphthalein.[49] From this point on cooling is not required.

Analysis of Filtrate.

Direct Colorimetric Method.—During the early part of this work the analyses were made, without any preliminary separation of the inorganic phosphate, by an adaptation of the colorimetric method of Fiske and Subbarow.[4] Colorimeter readings were taken 3 minutes after the addition of the reagents, and then at intervals of 1 minute for a sufficiently long period to permit the construction of a curve which could be extrapolated back to zero time. The sum of the phosphocreatine and inorganic phosphorus was computed from the reading obtained after the color intensity showed no further increase in relation to the standard, *i.e.* after 30 minutes or more, the exact time required for maximum color development depending on the temperature and other factors.

From numerous control analyses of known mixtures of phosphocreatine and inorganic phosphate, the most satisfactory direct colorimetric procedure for determining the concentration of inorganic phosphate has been found to be one based on only two readings, *viz.* those obtained after the expiration of 3 and 4 minutes, respectively. Under the assumption that the color intensity increases during the first 3 minutes at the rate found experimentally for the 4th minute, the calculation becomes a simple matter of arithmetic. For example, if the color intensities (in terms of mg. of phosphorus per 100 gm. of muscle) are found to be 45.2 at the end of the 3rd minute, and 51.1 at the end of the 4th, the inorganic

[49] If a saturated solution of sodium hydroxide is used to neutralize the filtrate the correction for the volume of the alkali is not more than 2 per cent, and for comparative purposes may be neglected. It is highly important to mix the slightly alkaline filtrate thoroughly before taking samples for analysis, for otherwise a uniform sample cannot be obtained on account of the precipitate (magnesium phosphate, *etc.*). In the case of resting muscle most of the inorganic phosphate may be in this sediment. Theoretically it would perhaps be better to take samples for analysis before the filtrate has been neutralized, but the acid solution will not keep for any length of time even in the cold, and we have experienced no trouble in getting uniform results in spite of the precipitate.

phosphate is estimated to be $45.2 - [3 \times (51.1 - 45.2)] = 27.5$ mg. of. P per 100 gm. of muscle. The maximum color in this instance was the equivalent of 87.0 mg. of P per 100 gm. of muscle, making the result for phosphocreatine P alone $87.0 - 27.5$, or 59.5 mg. per 100 gm.

The color production is not a single logarithmic reaction which is practically linear for the first few minutes, as is the case with relatively slow reactions of the first order. The approach to linearity is purely accidental. Actually there are two consecutive reactions, the first of which (the hydrolysis of phosphocreatine, many times accelerated by the presence of molybdate) is apparently unimolecular, while the second (the color reaction itself) is approximately of that order if the reagents are present in considerable excess. Taking the two reactions as a whole the course, except at the very outset, is for all practical purposes that of a single unimolecular reaction with a latent period. Whether or not the extrapolation method illustrated happens to give correct results depends on several factors, of which the most important is the temperature.[50]

In the use of this direct colorimetric method, frequently controlled by the analysis of mixtures of known composition, no trouble was experienced as long as the laboratory was heated artificially with thermostatic regulation, *i.e.* in winter. The data given in Table V show (1) that artificial mixtures of phosphocreatine and inorganic phosphate can be analyzed in this way with a fair degree of accuracy (Columns 4 to 7), (2) that the color

[50] Contrary to the statement made by Sacks and Davenport[51] this extrapolation method involves no assumption whatsoever regarding the mechanism of the reaction. It is merely an empirical procedure which gives satisfactory results when properly controlled against known mixtures, a test which Sacks and Davenport have apparently not considered it necessary to apply to their own analytical technique. As far as their attempts to use the direct colorimetric method are concerned there is unquestionably something seriously wrong, for the failure of the standard and unknown to match during the first few minutes after adding the reagents is an experience that we have never had with our reducing agent (whereas when hydroquinone is used it may be totally impossible to match the colors during the first few minutes). Nothing in the least suggestive of the "olive to blue-green" shades which Sacks and Davenport claim to have seen appears when the method is properly performed.

[51] Sacks, J., and Davenport, H. A., *J. Biol. Chem.*, **79**, 493 (1928).

production follows substantially the same course in muscle filtrates and in artificial mixtures (Columns 2 and 5), and (3) that the results are independent of the amount of filtrate (Columns 2

TABLE V.

Direct Colorimetric Method Applied to Muscle Filtrate and Known Mixtures.

Analyses of the muscle filtrate showed the presence of 27.4 mg. of inorganic P and 62.4 mg. of phosphocreatine P per liter. Solution A is an artificial mixture of this same composition, prepared from KH_2PO_4 and the secondary calcium salt of phosphocreatine. Solution B contains only half as much inorganic P, and Solution C none at all. The results are all expressed in mg. of phosphorus per liter (equivalent to 100 gm. of muscle). The colored solution was made up to 50 cc. in each experiment. Temperature, 20°.

Time.	Muscle filtrate (1 10).		Solution A.		Solution B.	Solution C.
	3 cc.	6 cc.	3 cc.	6 cc.	7 cc.	7 cc.
(1)	(2)	(3)	(4)	(5)	(6)	(7)
min.						
3	46 6	46.3	46.8	46.6	33.8	21.6
4	53.1	52.5	53.6	52.9	40.0	28.4
5	58.0	57.5	58.7	58.5	46.5	34.2
6	63.5	62.9	63.8	62.9	50.6	38.9
7	66.7	66.0	67.3	66.7	53.9	42.3
10	76 2	74 9	76.6	74.9	62.1	50.6
15	83 3	82.3	83.8	81.3	68.8	56.0
20	87.7	84.4	87.7	84.4	72.3	59.5
30	88.9	87.7	88.9	86.6	74.2	61.4
Maximum *	89.5	90.1	89.5	88.9	76.2	62.5
Inorganic P.						
Present.			27.4	27.4	13.7	0
Found† .. .	27.1	27.7	26.4	27.7	15.2	1.2
Phosphocreatine P.						
Present			62.4	62.4	62.4	62.4
Found	62.4	62.4	63.1	61.2	61.0	61.3

* Obtained by adding the molybdate-sulfuric acid mixture (Molybdate I)[4] and letting stand for 30 minutes before adding the reducing agent.

† Calculated from the readings made at 3 and 4 minutes, under the assumption that the color production is linear for the first 4 minutes.

and 3) or solution (Columns 4 and 5) used, provided of course that all the readings come within the range of proportionality.

Since the final color may be more than 3 times as intense as that

developed at the time of the first reading (3 minutes) the wide range of proportionality made possible by the use of aminonaphtholsulfonic acid[4] cannot be dispensed with.   In the hydroquinone method, provided that the reagents are added in sufficient excess to give the color reaction the required speed, the blank is so large that even with solutions containing only inorganic phosphate the results are unreliable unless the standard and unknown are of nearly the same strength.[4]   We have been unable to get even

TABLE VI.

Analysis of Phosphocreatine Solution at Different Temperatures.   Direct Colorimetric Method.

Phosphocreatine P present, 0.350 mg. Volume, 50 cc. Results expressed as per cent of theoretical maximum color.

Time. (1)	18° (2)	21° (3)	24° (4)
min.	*per cent*	*per cent*	*per cent*
3	28.9	38.9	50.1
4	39 3	51.1	64 2
5	48 6	61.7	73.7
6	56.6	69.7	80.5
7	63.9	75.2	84.6
10	78.8	86.6	92 3
15	89.6	92.9	96.9
20	94.5	96.0	97.7
30	96.9	96.9	97.7
Inorganic P.			
Present	0	0	0
Found*	−2.3	+2.3	+7.8

* Calculated as in Table V.

approximately correct results with hydroquinone on solutions of known composition, and consequently are wholly unable to agree on this point with Eggleton and Eggleton,[52] who are evidently quite unaware either of the defects of the Briggs method or of the effect of temperature on the course of the color reaction in solutions containing phosphocreatine.

In estimating the sum of the phosphocreatine and inorganic phosphate from the final reading a small error is involved.   Par-

[52] Eggleton, P., and Eggleton, G. P., *J. Physiol.*, **65**, 15 (1928).

ticularly if the color at the end of the reaction is considerably stronger than the standard—and in the analysis of resting muscle this should be the case in order that the initial readings may fall within the proper range—the theoretical maximum is never quite attained. Depending on the phosphate concentration, and to some extent also on the temperature, this error may be too small to be detected or it may be as much as 3 per cent of the total color. For many purposes the use of a small correction to offset this error may be accurate enough, but a better method is to determine the total color on a separate sample of the filtrate, which is allowed to stand for 30 minutes—or for whatever time is needed to give complete hydrolysis—after adding the molybdate. At the end of this period the standard is prepared in the usual way, and the reducing agent (aminonaphtholsulfonic acid) added as nearly as possible at the same time to both standard and unknown. The figures in Table V under the designation "Maximum" were obtained in this manner.

Analysis by the direct colorimetric method fails to give reliable results when the room temperature is high. Data on the progress of color development with a solution of pure phosphocreatine at 18°, 21°, and 24° are shown in Table VI. While the analysis of muscle filtrates under these particular conditions would be satisfactory at temperatures between 18° and 21°, the calculated concentration of inorganic phosphorus would be about 5 mg. per cent too high if the room temperature happened to be 24°. This merely serves to emphasize the fact that the method is entirely empirical, and should under no circumstances be used unless it has been controlled with known materials (*i.e.* either pure phosphocreatine, or at least muscle filtrates from which the inorganic phosphate has been precipitated, *e.g.* with calcium or barium hydroxide) at the same, or very nearly the same, temperature and with the same reagents.

. The speed of color production is not the same with all samples of ammonium molybdate. A comparison of Column 3, Table VI, with Column 2, Table VII will illustrate this point. The rate in the latter case is materially slower. Whether some preparations of ammonium molybdate contain an additional catalytic agent, or whether others contain impurity which acts in the opposite way, we cannot say. Once a solution of ammonium molybdate

and sulfuric acid (Molybdate I)[4] has been prepared, no evidence
that its properties will change in this respect over a period of
several weeks has so far appeared.

A relatively slight variation in acidity has a perceptible effect on
the rate of the reaction.   In all the experiments recorded in Tables
V and VI the concentration of sulfuric acid was 0.5 N, as prescribed
in the Fiske and Subbarow method for inorganic phosphate.[4]
From Table VII it will be evident that the development of color
is more rapid when the acidity is lower.   By empirical adjustment

TABLE VII.

Effect of Acidity.   Direct Colorimetric Method.

Phosphocreatine P present, 0.330 mg.   Volume, 50 cc.   Temperature,
21°.   Results expressed as per cent of theoretical maximum color.

Time. (1)	0.5 N H_2SO_4. (2)	0.4 N H_2SO_4. (3)	0.3 N H_2SO_4. (4)
min.	*per cent*	*per cent*	*per cent*
3	25.2	43.2	51.6
4	35.5	56.6	62.8
5	45.2	68 0	73.0
6	52.5	75.7	81.4
7	59.4	79.8	86.0
10	74.8	89.8	95.4
15	87.2	97.7	101.0
20	93.2	100.2	
30	96.2		
Inorganic P.			
Present.............	0	0	0
Found*.............	−5.7	+3.2	+17.9

* Calculated as in Table V.

of the acidity it is consequently possible to compensate for the
effect of temperature when necessary, and thus to adapt the
method to any circumstances as occasion may require.

The direct colorimetric method has been discussed at length
chiefly for the reason that in one form or another it has been used
in other laboratories without adequate control, whereas if results
of any value are expected a knowledge of the limitations of the
method is essential.   The direct procedure is still useful under
special circumstances, for the separation of the inorganic phosphate

by precipitation is sometimes not feasible (*e.g.* when the solution is excessively dilute), but for the routine analysis of muscle we have continued using it only so long as no other rapid method was available. The analytical scheme which we have finally adopted for muscle filtrates is the following.

Calcium Precipitation Method.—In any method involving the separation of the inorganic phosphate by precipitation, if it is to be applicable without material modification to all sorts of filtrates, provision should be made for the direct determination of the phosphocreatine. If the concentration of phosphocreatine is very low, as in stimulated muscle for example, the difference between the total color given by the filtrate as it stands and the color obtained from the precipitated inorganic phosphate may be within the experimental error. For the same reason analysis by difference may indicate the presence of a small amount of phosphocreatine when none at all is present. Precipitation with magnesium citrate mixture is accordingly not suitable for general use. In the first place, analysis of the filtrate (or supernatant fluid) is interfered with by citrate and ammonium salts, which delay the color reaction.[4] Secondly, the precipitation of magnesium ammonium phosphate from solutions containing much less than 100 mg. of inorganic phosphorus per liter is likely to be extremely slow, and from very dilute solutions may be incomplete even after several hours standing.[27] Finally the triple phosphate, like most crystalline precipitates, tends to float on the surface of the liquid, so that some loss is almost certain to occur if the centrifuge is used. This source of error may of course be obviated by separating the precipitate with a micro filter of some kind which can be washed with a small amount of fluid, but the results, however accurate they may be under the best conditions, hardly justify the time and trouble required to make the magnesium method universally reliable.

Precipitation of the inorganic phosphate as the calcium salt, on the contrary, is almost instantaneous. (As a matter of routine we let the mixture stand for 10 minutes to permit the precipitate to become agglutinated.) This method is consequently a relatively rapid one, and is carried out as follows:

4 cc. of the neutralized (and thoroughly mixed) trichloroacetic acid filtrate are pipetted into a centrifuge tube and treated with

1 cc. of a 10 per cent solution of $CaCl_2$ which has been saturated
with calcium hydroxide. After 10 minutes standing the suspen-
sion is centrifuged for 2 minutes at about 3000 R.P.M., the super-
natant fluid poured out into a volumetric flask or graduated
test-tube of suitable size, and the sediment washed on the centri-
fuge with a mixture of 4 cc. of water and 1 cc of the same $CaCl_2$-
$Ca(OH)_2$ solution. The washings are added to the original
supernatant fluid, and the combined solutions then used for the
direct determination of the phosphocreatine. The proper size
of the volumetric flask (or test-tube) will vary from 50 cc. in
the case of normal resting muscle to 15 cc. when the phospho-
creatine content is very low. If a 50 cc. flask is to be used, 25
cc of water are first added to contents, followed by 5 cc. of Molyb-
date I (2.5 per cent ammonium molybdate in 5 N sulfuric acid).[4]
After half an hour (or longer if the temperature is low or the
reaction delayed for any other reason) the color is developed by
the addition of 2 cc. of the aminonaphtholsulfonic acid solution,
and the usual standard[4] prepared as nearly as possible at the same
time. (Ordinarily the analysis of the calcium precipitate will of
course be carried out simultaneously.)

When the concentration of phosphocreatine is so low that the
colored solution must be diluted to 15 or 25 cc. (instead of 50) a
slight change in the procedure must be made, for the concentration
of calcium sulfate in the solution will then exceed its solubility.
The calcium sulfate crystallizes slowly, and the separation will
not be complete if the mixture is left undisturbed for 30 minutes,
with the result that crystals will appear on the walls of the colorim-
eter cup, spoiling the analysis. A very simple remedy for this
is to seed the solution with a few crystals of calcium sulfate
($CaSO_4 \cdot 2H_2O$) immediately after the addition of Molybdate I.
If the contents of the flask or test-tube are then thoroughly mixed
once by rotation, crystallization will be complete by the time the
phosphocreatine is hydrolyzed. After addition of the reducing
agent and dilution to the mark, it is a simple matter to remove
the calcium sulfate crystals by centrifugation.

For the determination of inorganic phosphate the washed cal-
cium phosphate precipitate is dissolved with a drop or two of N
sulfuric acid, and the contents of the centrifuge tube rinsed into a
25 or 50 cc. volumetric flask. The color is developed in the usual
way at the same time that the standard is prepared.

The following formula may be used for all the calculations.

$$\text{Mg. P per 100 gm. muscle} = \frac{D\,(4V - 3w)}{2Rw},$$

where w is the weight of the muscle sample (in gm.), V the volume of the trichloroacetic acid solution used (in cc.), D the volume of the measuring flask or graduated test-tube (in cc.), and R the colorimeter reading (the standard being set at 20 mm.). This formula allows for the water in the muscle itself, which is assumed to contain 75 per cent of moisture. The slight variations in the

TABLE VIII.
Analysis of Known Mixtures. *   Calcium Precipitation Method.*

Phosphocreatine P.		Inorganic P.	
Present.	Found.	Present.	Found.
mg. per l.	*mg. per l.*	*mg. per l.*	*mg. per l.*
79.2	78.3	13.7	13.7
63.4	62.5	27.4	27.3
31.7	31.8	68.5	69.0
15.8	17.1	82.2	82.0
7.9	9.3	95.9	95.0
4.0	4.4†	95.9	96.2

* Mixtures of KH_2PO_4 and phosphocreatine (Ca salt) in 5 per cent sodium trichloroacetate.

† Precipitate not washed.

water content of muscle which occur under ordinary circumstances may be disregarded.

When the phosphocreatine content of muscle is comparatively low the results for this substance obtained by the calcium precipitation method come out about 1 mg. of P per 100 gm. too high (Table VIII). Under these conditions somewhat greater accuracy may be secured if the calcium phosphate sediment is not washed, with the added advantage that it is then possible to get a stronger color, for the final volume need not be more than 10 cc. A still better method, especially when the phosphocreatine is very low, is to filter off the calcium phosphate (instead of centrifuging it), using an aliquot portion of the filtrate for the determination of the phosphocreatine. In this case the precipitate is not analyzed.

but the sum of the inorganic phosphate and the phosphocreatine determined by means of the direct colorimetric method on a separate sample of the muscle filtrate, and the inorganic phosphate estimated by difference. For example, an artificial mixture containing 95.6 mg. of inorganic P and 2.63 mg. of phosphocreatine P per liter was analyzed by precipitating 16 cc. with 4 cc. of the $CaCl_2$-$Ca(OH)_2$ solution, filtering, and determining the phosphocreatine in 8 cc. of the filtrate (equivalent to 6.4 cc. of the original solution). The color was developed in a test-tube marked at 10 cc., and read against the customary standard. The reading was 46.5 mm., which corresponds to 0.0172 mg. of phosphorus, or 2.69 mg. per liter (as compared to the 2.63 mg. actually present). For th› total color, 1 cc. of the same mixture in a 25 cc. volumetric flask read 20.5, which gives 97.6 mg. per liter for the sum of the inorganic and phosphocreatine P, or 97.6 − 2.7 = 94.9 mg. of inorganic phosphorus (instead of 95.6).

If the high results for phosphocreatine when the centrifuge is used are caused by incomplete sedimentation of the inorganic phosphate, as we suppose must be the case, it appears that a small amount of calcium phosphate is so finely divided that it remains suspended even after 2 minutes centrifugation at 3000 R.P.M., although it is retained by filter paper of comparatively loose texture (Schleicher and Schüll No. 604), as well as by Jena filters of sintered glass. Since perfectly satisfactory results can be obtained by filtration when the phosphocreatine is low, and since analyses of artificial mixtures which correspond in composition to filtrates from resting muscle (see the first three analyses of Table VIII) indicate that the centrifuge method is accurate enough under these conditions (perhaps because of compensating errors), further modification has not been attempted.

Phosphocreatine and Inorganic Phosphate in Voluntary Muscle of Cat under Various Conditions.

Illustrative analytical data on the composition of the resting muscle of the cat (anesthetized with amytal), and on the effect of ischemia, postmortem change, stimulation, and recovery, are contained in Tables IX to XI, which are to a large extent self-explanatory.

A certain amount of twitching is practically always perceptible

TABLE IX.

Normal Resting Muscle.

Name of muscle.	Phospho-creatine P.	Inorganic P.	Sum.
	mg. per cent	*mg. per cent*	*mg. per cent*
Adductor brevis..............................	75	23	98
Extensor longus digitorum.*................	64	27	91
" " " *................	86	35	121
Flexor longus digitorum.....................	65	27	92
Gastrocnemius..............................	70	20	90
"	69	23	92
"	55	28	83
Gracilis*...................................	58	23	81
" *.................................	51	33	84
Rectus femoris.............................	62	28	90
" "	58	32	90
Sartorius...................................	81	18	99
" *...................................	62	24	86
"	55	27	82
Vastus lateralis............................	74	22	96
" "	62	25	87

* Liquid air.

TABLE X.

Effect of Lack of Circulation.

Treatment.	Name of muscle.	Phospho-creatine P.	Inorganic P.	Sum.
		mg. per cent	*mg. per cent*	*mg. per cent*
Artery clamped 10 min............	Gracilis.	38	44	82
" " 30 "	Biceps.	18	72	90
" " 80 "	Gastrocnemius.	8	69	77
" " 120 "	Gracilis.	20	60	80
Artery and vein clamped 30 min....	Biceps.	9	87	96
" " " " 30 "	"	10	85	95
" " " " 30 "	"	23	62	85
10 min. post mortem................	Gastrocnemius.	30	60	90
20 " " "	"	8	78	86
30 " " "	Biceps.	0	82	82

when the muscle is removed from its surroundings. The loss of phosphocreatine and gain in inorganic phosphate during the preparation of the sample are consequently bound to vary, depending on the irritability of the muscle, the nature of its insertions, *etc.* However, unless the muscle goes into violent

TABLE XI.
Stimulation and Recovery.

Stimulation.	Recovery.	Name of muscle.	Phospho-creatine P.	Inorganic P.	Sum.
Intermittent tetanization of nerve.					
			mg. per cent	*mg. per cent*	*mg. per cent*
10 min.		Gastrocnemius.	24	65	89
25 "		"	34	39	73
25 "		Biceps.	21	66	87
Continuous tetanization of nerve.					
5 sec.		Gastrocnemius.	33	52	85
10 "		"	29	54	83
20 "		"	24	58	82
3 min.		"	17	55	72
6 "		"	16	57	73
30 "		"	11	69	80
9 "		Biceps.	2	84	86
12 "		"	0	82	82
Completely fatigued with artery clamped.					
4 min.		Biceps.	0	77	77
4 "		Gastrocnemius.	0	70	70
4 "		"	0	74	74
Recovery (after complete fatigue) with circulation restored.					
	25 min.	Biceps.	41	28	69
	25 "	"	41	27	68
	1 hr.	Gastrocnemius.	46	19	65

contraction while it is being taken out, the phosphocreatine is generally found to be 70 to 75 per cent of the sum of the two constituents (Table IX). While there is hardly any question that the inorganic phosphorus content of the muscle in its natural surroundings is lower than indicated by the analytical results,

and may therefore be well under 20 mg. per cent in full relaxation, figures in excess of 30 mg. per cent are sometimes found, even when no more than the usual activity during the isolation of the muscle is in evidence. We accordingly believe that in spite of the anesthesia some variation in the physiological condition of the tissue, reflected in its inorganic phosphate content, must be reckoned with in the case of muscle which is ostensibly in the resting state before it is dissected out. In investigating the effect of experimental procedures it appears therefore unwise to conclude that the inorganic phosphorus has been increased unless the result exceeds 35 mg. per cent.

With regard to the recently published work of Sacks and Davenport[51] on muscle frozen gradually *in situ*, we should like to call attention to the fact that their two analyses of cat muscle (21 and 22 mg. per cent, respectively, of inorganic phosphorus) are no lower than several of our own obtained with muscle which was excised before cooling. Since 20 mg. per cent is almost certainly too high for completely relaxed muscle, we are not prepared to concede that the technique used by Sacks and Davenport definitely establishes "the normal range of inorganic phosphate." We are also unable to follow the logic of their argument that the composition of muscle subjected to this distinctly artificial treatment is necessarily identical with that of the normal living tissue. Their very low results for lactic acid[53] are certainly of interest, and may mean that the "lactic acid minimum" for mammalian muscle is much less than was formerly supposed. But it is also possible that during the slow cooling process equilibrium is reestablished on a new basis, and that resting muscle under *natural* conditions contains more lactic acid than these investigators find.

Hydrolysis of phosphocreatine takes place rather rapidly when the circulation is shut off (Table X), but some may still be present after 2 hours with no blood supply. Decomposition is complete in a much shorter period when the animal is dead.

The stimulation of muscle by uninterrupted tetanization of the

[53] *Cf.* also Davenport, H. A., and Davenport, H. K., *J. Biol. Chem.*, **76**, 651 (1928). Davenport, H. A., Davenport, H. K., and Ranson, S. W., *J. Biol. Chem.*, **79**, 499 (1928).

nerve soon destroys a large proportion of the phosphocreatine even when the circulation is intact (Table XI). Whether or not the disappearance is complete apparently depends upon the muscle used. The gastrocnemius, for example, unless the artery is clamped, contains 10 mg. or more of phosphocreatine phosphorus per 100 gm. even after long continued stimulation, and in our experience cannot be entirely fatigued. The biceps (brachii) on the other hand loses its irritability completely in the course of a few minutes stimulation, and the phosphocreatine content falls practically to zero. Other instances of correlation between the presence of phosphocreatine and ability to contract have been observed in experiments with surviving muscle.

Isolated cat muscle suspended in oxygenated Ringer's solution at body temperature soon loses its ability to contract on stimulation, and under the same conditions the phosphocreatine is rapidly destroyed (in half an hour or less). In more favorable media the irritability may be retained for 2 hours or more, and sometimes for as long as 6 hours. Tyrode's solution buffered with 0.1 M glycine and adjusted to pH 7.4 has given the best results in our hands. In twelve experiments of this type the phosphocreatine content at the end of 2 hours varied between 7 and 26 mg. of P per 100 gm. of muscle (average 18 mg.), while in four experiments of 5 to 6 hours duration we found from 9 to 20 mg. per cent (average 15 mg.). In a few instances the fact that the muscle was still irritable was established by the briefest possible electrical stimulation at the conclusion of the experiment, a procedure which would naturally cause a portion of the remaining phosphocreatine to be destroyed, and so was generally omitted. However, in some of the experiments in which this test was not applied the muscle contracted of its own accord at intervals. On the other hand, in two experiments with muscle suspended in oxygenated Tyrode's solution without the glycine buffer, a stimulus applied after 90 minutes failed to elicit a response, and in these no phosphocreatine could be detected.

The muscles used in the above experiments (performed by Mr. A. E. Hardy) were the extensor longus digitorum and the extensor carpi ulnaris, weighing generally between 2 and 4 gm., and suspended in 20 to 25 cc. of solution.

Several attempts were made to bring about the resynthesis of phosphocreatine in cat muscle which had first been completely

fatigued *in situ* with the blood supply cut off.   After 2 hours in Tyrode's solution (buffered with glycine) at 37° the results were invariably negative.   Occasionally it has been possible to demonstrate the formation of a small amount of phosphocreatine (7 to 16 mg. of P per 100 gm. of muscle) in previously fatigued muscle when adrenaline is added (0.25 to 0.5 cc. 1:1000, mixed with the salt solution at the beginning of the experiment or added in four installments during the 2 hour period).   Even with adrenaline, however, frequently no synthesis occurs.

The remaining experiments in Table XI call for little comment. They show (1) that stimulation to complete fatigue (about 4 minutes) after shutting off the blood supply destroys all the phosphocreatine, and (2) that a certain amount of resynthesis occurs in muscle which has been so treated if it is allowed to rest for a time after removing the arterial clamp.   In one experiment not included in Table XI 20 mg. of phosphocreatine phosphorus (per 100 gm. of muscle) were found after a recovery period of 1 hour with the circulation not restored.

Partial hydrolysis of phosphocreatine *in vivo* can be brought about in other ways.   Thus, after the intravenous injection of potassium chloride (50 mg. per kilo) the inorganic phosphate content of the muscle is increased and the phosphocreatine correspondingly diminished (Table XII), a result which may have some bearing on the mechanism of potassium paralysis.[54]   Lactic acid has a similar effect.   This might be taken as an indication that the process of phosphocreatine hydrolysis is set in operation during muscular contraction by lactic acid; but if so the predominance of lactic acid production can be only momentary, for experiments with frog muscle have clearly shown that during the first 5 seconds tetanus twice as much inorganic phosphate as lactic acid is produced.[55]

[54] Because of the effect of potassium on the heart, limiting the dose that can be given to the living animal, we have attempted to repeat these experiments on muscle isolated from the circulation.   After the aorta and the vena cava had been tied and cut, about 200 cc. of a solution of potassium chloride (0.1 to 0.5 per cent) were injected through the artery.   The results, however, not only with potassium chloride but also with various other substances tried following the same technique, were so irregular in spite of numerous controls that the method has been discarded as unsuitable.

[55] See Meyerhof, O., and Lohmann, K., *Naturwissenschaften*, **15**, 670 (1927).

Thinking that perhaps the fatigue products (lactic acid, *etc.*) liberated from one group of contracting muscles and distributed by the blood stream to other portions of the body might induce hydrolysis of phosphocreatine in muscles which for the time being are not active, we have put this question to the experimental test. The nerve trunk supplying one hind leg was cut, and the peripheral end stimulated with a continuous tetanizing current until there was no further perceptible decrease in the response. Analysis of

TABLE XII.

Miscellaneous Experiments with Cats.

Experimental procedure.	Name of muscle.	Phos-pho-creatine P.	Inor-ganic P.	Sum.
		mg. per cent	*mg. per cent*	*mg. per cent*
Creatine (300 mg. per kilo) plus 1 equivalent Na₂HPO₄ intravenously.	Gracilis.	50	32	82
" "	Rectus femoris.	53	34	87
Potassium chloride (50 mg. per kilo) intravenously.	Gastrocnemius.	36	48	84
" "	Biceps.	32	50	83
Lactic acid (20 mg. per kilo) intravenously.	Triceps.	22	50	72
" "	Gastrocnemius.	24	53	77
Prolonged stimulation of peripheral cut end of nerve trunk supplying opposite leg.	"	54	32	86
" "	"	54	37	91

the gastrocnemius from the opposite leg however failed to show unmistakable evidence of change[56] (Table XII).

Finally, it has proved to be impossible to increase the phosphocreatine content of the muscles by the simultaneous injection of creatine and disodium phosphate, at least in acute experiments. In the examples shown in Table XII the amount of creatine injected was 300 mg. per kilo of body weight, together with 1 equivalent of phosphate. Similar results have also been obtained

[56] The results (32 and 37 mg. of inorganic phosphorus) are indecisive one way or the other.

with creatine alone, and with creatine mixed with 10 equivalents of phosphate.

Relation to Previous Work.

The first satisfactory experimental evidence that muscle creatine *in vivo* is not free creatine is to be found in the work of Folin and Denis,[57] who showed in 1914 that free creatine, injected into the circulation or absorbed from the digestive tract, accumulates in the muscle against an apparent concentration gradient. 7 years earlier Urano,[58] on the basis of dialysis experiments with isolated muscle, had suggested the existence of a creatine-containing complex, but from what has now been learned of the properties of phosphocreatine the explanation of this author's findings must be sought in some other quarter.

Urano found, in substance, that creatine diffuses out of dog muscle (suspended in cold Ringer's solution) at a considerably higher rate if the tissue has first been kept on ice for 1 or 2 days than in the case of fresh material—in the early part of the experiment from 4 to 6 times as rapidly. The muscle samples however were cut into strips beforehand, and the strips tied together into bundles. This treatment is in itself sufficient to destroy a large part of the phosphocreatine, and it is quite impossible that the concentration of free creatine could be from 4 to 6 times as high in one case as in the other. Moreover, the more rapid diffusion from muscle which had been allowed to stand on ice was in evidence even after 16 hours dialysis, and in one experiment was most marked in the second 2 hour period.

Actually, within the error of Urano's analyses—for which no colorimeter was used—the concentration of free creatine has ceased to change before the first 2 hours have elapsed when muscle is immersed in Ringer's solution at 0°. Of three samples of dog muscle which we have treated as nearly as possible according to the technique used by Urano (cutting into strips, *etc.*) the one showing the least marked decomposition in the fresh state contained only 26 mg. per cent of phosphocreatine P, and after standing in ice-cold Ringer's solution for 2 hours only 6 mg. per cent remained. By subtracting the creatine equivalent of these figures (P × 4.22) from the total creatine content the concentrations of free creatine (including preformed creatinine) were found to be 324 and 400 mg. per cent, respectively. In another portion of the same muscle kept for 48 hours in the cold room the free creatine increased only 31 per cent (to 425 mg. per cent), a change far too slight to account for Urano's results on the basis of his own interpretation.

[57] Folin, O., and Denis, W., *J. Biol. Chem.*, **17**, 493 (1914).

[58] Urano, F., *Beitr. physiol. u. path. Chem.*, **9**, 104 (1907).

Urano's single experiment in which the diffusion of the total phosphorus was followed is still less to the point. In this case the rate of diffusion from the muscle which had been kept on ice (for 2 days) was approximately twice as great during each of the first three 2 hour periods, *i.e.* for a total of 6 hours.

The results of these dialysis experiments are therefore in all probability to be accounted for by some change in the physical properties of the cell membrane. Urano in fact considered the possibility that an alteration in permeability might be the controlling factor, but he dismissed it with the rather curious statement that the sarcolemma is not known to undergo postmortem change.

Whether the discovery of phosphocreatine provides a complete explanation of the experiments of Folin and Denis is an open question, inasmuch as there is still some doubt as to the concentration of free creatine in living voluntary muscle under natural conditions. In trichloroacetic acid filtrates prepared as described above for phosphate determinations (except that the indicator is omitted) we have found on the average about 140 mg. of creatine (per 100 gm. of muscle) in excess of that accounted for as phosphocreatine on the supposition—which is surely not correct—that no phosphocreatine is hydrolyzed during the preparation of the sample. At the other extreme, if the assumption is made that all the phosphorus present in the protein-free filtrate as inorganic phosphate has also been combined with creatine in the living and completely relaxed muscle, the concentration of free creatine is only about 30 mg. per cent. The experiments of Baumann and Ingvaldsen[31] however show that the direct colorimetric determination of creatine in muscle extracts frequently gives results as much as 30 mg. per cent higher than those obtained when the colorimetric analysis is preceded by isolation as creatinine potassium picrate. It is consequently possible that that much of the chromogenic substance is not creatine at all.

If the inorganic phosphate content of living unstimulated muscle is very small it may therefore be that little or no free creatine is present, in which case the existence of phosphocreatine would suffice to explain the observations of Folin and Denis. If, on the other hand, the hydrolysis incident to the preparation of the muscle sample is slight, and the concentration of free creatine in resting muscle accordingly not far below 140 mg. per cent, it appears unlikely that this substance would be absorbed by muscle

from the circulation to the extent that has been found.   Further information is obviously needed.

Within the last few years two series of experiments, in some respects similar to those of Urano, have been reported.   In 1922 Embden and Adler[59] showed that frog muscle, suspended in Ringer's solution, loses phosphoric acid very slowly in the resting condition, but much more rapidly when it has been fatigued. 3 years later Tiegs[60] found the same to hold for creatine.   While Tiegs attempted to explain his findings by assuming, on grounds which from the chemical standpoint are entirely inadequate, that resting muscle contains a non-dialyzable tautomer of creatine, the interpretation offered by Embden and Adler[61,59] was quite different.   According to their hypothesis fatigue is accompanied by an increase in the permeability of the membrane around the muscle fiber, and on this basis they devised a theory of muscular contraction.   The essential feature of the Embden-Adler theory is that a sudden increase in the permeability of the cell membrane is an integral part of the response to stimulation.   In completely fatigued muscle the permeability is supposed to be already at its maximum, and since no further increase is then possible contraction is unable to occur.

Inasmuch as frog muscle, unlike mammalian muscle, survives for a considerable period under such conditions, and since the muscles were not cut, there appears to be no doubt that the results of Embden and Adler with phosphate, as well as those of Tiegs with creatine, are at least partially explained by the fact that resting muscle contains a large amount of phosphocreatine, whereas in fatigued muscle this substance has been hydrolyzed.   The recent work of Stella[62] in fact indicates that for a given concentration gradient inorganic phosphate neither enters nor escapes from muscle more rapidly in the fatigued state.   From this result, taken in conjunction with the experiments of Embden and Adler and of Tiegs, it follows that frog muscle is—at least relatively— impermeable to phosphocreatine and that Tiegs was consequently right in ascribing the difference between resting and fatigued

[59] Embden, G., and Adler, E., *Z. physiol. Chem.*, **118**, 1 (1922).

[60] Tiegs, O. W., *Australian J. Exp. Biol. and Med. Sc.*, **2**, 1 (1924–25).

[61] Embden, G., *Ber. ges. Physiol. u. exp. Pharmakol.*, **2**, 159 (1920).

[62] Stella, G., *J. Physiol.*, **66**, 19 (1928).

muscle to a chemical change involving creatine, even if he was entirely mistaken in the premises which led him to make this suggestion and in the nature of the change.[63]

SUMMARY.

Various methods are described for the isolation of phospho-creatine from protein-free muscle filtrates. In most cases the yields are very poor, but by one method 70 per cent of the labile phosphorus present in the protein-free filtrate can be recovered as a crystalline calcium salt. This product, which is a mixture of the secondary and tertiary salts, can be converted to the pure secondary salt, $C_4H_8O_5N_3PCa \cdot 4H_2O$. From the latter, after hydrolysis, practically the theoretical amount of creatine can be obtained. The constitution of phosphocreatine is therefore probably as follows:

$$\text{IIN} : \text{C} \begin{cases} \text{NH} \cdot \text{PO(OH)}_2 \\ \\ \text{N(CH}_3) \cdot \text{CH}_2 \cdot \text{COOH} \end{cases}$$
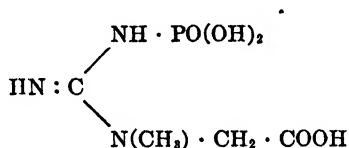
Because of the stimulation incident to the removal of large masses of muscle on the scale required for the preparation of phosphocreatine, the protein-free filtrate contains only about two thirds of the labile phosphorus originally present in the muscle. This is all phosphocreatine, for the 30 per cent loss in the succeeding steps of the isolation occurs also in control experiments with known amounts of the pure substance. The labile phosphorus

[63] As for the Embden-Adler theory itself, the one remaining fact which seems to favor it is that chloride also diffuses out more rapidly when the muscle is fatigued (Embden, G., and Lange, H., *Z. physiol. Chem.*, **130,** 350 (1923); *Klin. Woch.*, **3,** 129 (1924). Embden, G., Handbuch der normale und pathologischen Physiologie, Berlin, **8,** 413 (1925)). In the case of lactic acid however the reverse is true.[64] Particularly in consideration of the fact that the theory can no longer derive support from the experiments (with phosphate) on which it was originally based the bulk of the evidence appears to be against it. It has recently been pointed out that the rate of diffusion from the muscle may for that matter have no relation whatsoever to the permeability of the cell membrane. [62,64]

[64] Eggleton, G. P., Eggleton, P., and Hill, A. V., *Proc. Roy. Soc. London, Series B.*, **103,** 620 (1928).

compound in the protein-free filtrate hence contains nothing but creatine and phosphoric acid. The physiological significance of the physicochemical properties of phosphocreatine is nil without knowledge of this fact.

The second acid dissociation constant of phosphocreatine, determined by the titration of 0.005 M solutions of the secondary calcium salt with acid, is 2.6×10^{-5}. Since the method of intravital staining with indicators has shown the interior of the muscle fiber to be distinctly acid (Rous), and since the labile phosphorus of muscle has now been shown to be phosphocreatine itself (and not some substance of which phosphocreatine is a decomposition product), it follows that the hydrolysis of this substance during muscular contraction is accompanied by the liberation of a large amount of base, and consequently functions as a mechanism for neutralizing acid.

The rate of hydrolysis of phosphocreatine by acid in a mixture of the primary and secondary salts is proportional to the mol fraction of the primary salt. In other words the primary salt is hydrolyzed by water; the secondary salt is not.

Two methods are given for the determination of phosphocreatine (and inorganic phosphate) in muscle. The first is a direct colorimetric method, which is affected by the temperature and other factors, and is consequently capable of giving accurate results only when properly controlled with known solutions. The second method is based on a preliminary separation of the inorganic phosphate by means of calcium and is more reliable, particularly when the tissue contains only a small amount of phosphocreatine.

Analytical data are presented showing the composition of normal resting muscle (cat), as well as the hydrolysis of phosphocreatine during contraction and after shutting off the circulation, and its resynthesis during recovery. Hydrolysis has been demonstrated also after the intravenous injection of lactic acid and of potassium chloride.

Intravenous injections of creatine (alone or mixed with sodium phosphate) do not increase the concentration of phosphocreatine in muscle.

It has so far been impossible to show conclusively that the contraction of one set of muscles induces the hydrolysis of phosphocreatine elsewhere in the body.

ALKALINE RESERVE AND OXYGEN CAPACITY OF ARTERIAL AND OF VENOUS BLOOD.

By L. M. HURXTHAL, A. V. BOCK, J. H. TALBOTT, and D. B. DILL.

(From the Medical Laboratories of the Massachusetts General Hospital, Boston.)

Under certain conditions the alkaline reserve, or carbon dioxide capacity, of arterial blood may differ considerably from that of venous blood. Thus Barr and Himwich (1) found that during and after severe muscular work of 3 to 5 minutes duration on the bicycle ergometer, the carbon dioxide capacity of arterial arm blood might be 3 or 4 volumes per cent less than that of venous arm blood. Consistent with this observation was their finding that lactic acid disappears from blood as it passes through the resting arm and their conclusion that recovery from short bouts of exhausting work may in part take place in muscles not directly involved in the work.

Peters, Bulger, and Eisenman (2) have reviewed the literature on this subject. They examined both normal blood and blood in various pathological conditions and found considerable differences between arterial and venous blood. In one normal subject there were 3 volumes per cent more carbonic acid in venous than in arterial blood. This difference is 15 times the ordinary limits of error in blood gas analysis. Most of the subjects employed in their investigation were "chosen because it seemed likely that they would present maximum *venous-arterial* differences." Fraser, Graham, and Hilton (3) also report considerable differences in bicarbonate content in arterial and venous blood in both normal and pathological subjects.

` During the past 3 years, incidental for the most part to other experiments, we have collected much information on this same question. While this has in part been published in other papers from this laboratory and by L. J. Henderson (4), it has all been collected for the first time as shown in the accompanying Table I.

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TABLE I.

Alkaline Reserve and Oxygen Capacity of Arterial and of Venous Blood.

Subject.	Date.	Remarks.	Total CO₂ at pCO_2 = 40 mm.			Oxygen capacity.		
			Arterial blood.	Venous blood.	Δ total CO₂.	Arterial blood.	Venous blood.	
A. Normal subjects at rest.								
	1926		vol. per cent	vol. per cent	vol. per cent	vol. per cent	vol. per cent	v 1 c
J. S. L.	Feb. 12	After lunch.	49.3	48.4	+0.9	20.4	19.8	+
	1927							
J. C.	Feb. 23	Basal.	48.8	47.6	+1.2	21.2	21.2	
J. H. T.	Nov. 16	"	46.9	46.4	+0.5		21.8	
	1928							
D. B. D.	Sept. 18	Basal.	50.2	50.6	−0.4	18.4	18.7	−
A. W. M.	Oct. 22	"	49.6	48.6	+1.0	20.2	20.7	−
W. J. G.	" 24	Not basal.	49.2	49.2	0.0	22.2	22.2	
Average Δ					+0.5			
B. Normal subjects working in a steady state.								
	1926	Oxygen used. *l. per min.*						
A. V. B.	Jan. 19	1.2	47.3	47.7	−0.4	21.1	20.9	+
"	" 26	1.8	36.9	37.9	−1.0	22.3	22.5	−
"	Feb. 3	1.5	39.9	41.2	−1.3	21.3		
"	" 17	1.8	40.0	40.8	−0.8	21.9	21.8	−
L. M. H.	Jan. 14	1.9	42.2	42.7	−0.5			
"	Feb. 9	1.7	43.0	43.5	−0.5	21.3	22.1	+
D. B. D.	" 11	1.7	43.8	44.3	−0.5		19.2	
Average Δ..					−0.7			+

TABLE I—*Concluded*.

Subject.	Date.	Remarks.	Total CO$_2$ at pCO$_2$ = 40 mm.		Δ total CO$_2$.	Oxygen capacity.		Δ oxygen capacity.
			Arterial blood.	Venous blood.		Arterial blood.	Venous blood.	

C. Pathological subjects.

Subject.	Date. 1927	Diagnosis.	vol. per cent	vol. per cent	vol. per cent	vol. per cent	vol. per cent	vol. per cent
McD.	Jan. 4	Leukemia.	40.0	39 2	+0.8	6.1	6.7	−0.6
A. H.	" 11	Myxedema.	51.0	49 2	+1.8	17.1	17.2	−0.1
E. M.	" 15	"	48.6	49.3	−0.7	11.9	12.1	−0.2
Tr.	" 22	Terminal nephritis.	22.1	22.3	−0.2	8.4	8.5	−0.1
J. F. J.	" 28	Pernicious anemia.	60.7	59.1	+1.6	6.2	7.0	−0.8
"	Mar. 1	Recovering.	53.9	53.8	+0.1	12.4	12.5	−0.1
"	" 23	"	50.1	49.8	+0.3	15.4	16.0	−0.6
B.	Feb. 14	Myxedema.	39.8	38.5	+0.8	20.0	20.7	−0.7
T. B.	Sept. 9	Diabetic coma.	16.5	16.5	0.0		22.0	
P. J.	Oct. 21	Hypertension.	46.5	45.8	+0.7	22.1		
Ro.	Nov. 22	Nephritis.	31.7	31.7	0.0	7.1	7.4	−0.3
Co.	" 27	"	25.1	26.8	−1.7	12.9	12.9	0.0
R.	Dec. 9	"	27.9	30.2	−2.3	7.0	7.3	−0.3
	1928							
H. L.	Apr. 17	Cretin; thyroid therapy.	46.3	45.9	+0.4	20.6	21.5	−0.9
M. B.	" 17	Myxedema; thyroid therapy.	44.2	47.0	−2.8	16.2	16.5	−0.3
Average Δ...					−0.1			−0.4

Since most of the methods used in equilibration and analysis have been described elsewhere (4), we need not repeat the description here. The level of the carbonic acid dissociation curve when the partial pressure of carbon dioxide is 40 mm. was determined by equilibrating whole blood at pressures below and above this point and interpolating on the line determined by plotting log pCO$_2$ against log (total CO$_2$)$_B$ as suggested by Peters (5). Only experiments are included in which the curve for arterial blood was approximately parallel to that of venous blood as well as consistent with hemoglobin concentration and carbonic acid capacity.

The results require little amplification. It appears that in normal subjects and in the majority of our pathological subjects the alkaline reserve of arterial blood is slightly higher than that of venous blood, while in exercise in the steady state the reverse is the case. In twenty-one experiments on resting subjects, normal and pathological, the difference exceeded 1 volume per cent in six cases and 2 volumes per cent in two cases, the greatest difference being 2.8 volumes per cent. In seven experiments on subjects riding in a steady state for 15 or more minutes on the bicycle ergometer, blood being taken while work continued, the alkaline reserve of arterial blood was less than that of venous blood in every case, the greatest difference being 1.3 volumes per cent. This difference is qualitatively the same as that found by Barr and Himwich (1). Their differences were greater than ours, probably because the bouts undergone by their subjects were shorter and more exhausting than those of our experiments.

Our nineteen observations on oxygen capacity of arterial and venous blood include only three in which the oxygen capacity of arterial blood was greater than that of venous blood and none in which the difference was as great as 1 volume per cent in either direction.

It is evident that in normal subjects at rest and in moderate work and in many pathological conditions the difference between arterial and venous blood is for most purposes unimportant. It is true that values for respiratory quotient and for rate of blood flow found by examination of simultaneous samples of arterial and venous blood from the arm are unreliable. If one determines (a) rate of blood flow by one of the established indirect methods, (b) respiratory quotient by analysis of expired air, and (c) partial pressure of carbon dioxide in alveolar air, the results can be applied to the carbon dioxide and oxygen dissociation curves of venous blood without much chance of error. One must except, of course, extreme conditions such as decompensated heart disease and the exhaustion following a short period of severe exercise.

A fair question yet remains to be answered. Do the small differences observed by us and the much larger differences observed under extreme conditions by Barr and Himwich as well as by Peters, Bulger, and Eisenman represent real differences in the circulating blood? Obtaining venous blood under novocaine

anesthesia is usually a painless procedure.   Even so in one apprehensive individual we have observed a drop of 10 mm. in the partial pressure of carbon dioxide in alveolar air during venepuncture.   Not uncommonly a tourniquet is used before the puncture.   This is removed as soon as puncture is effected but there is possibly a slight effect on the blood nevertheless.   Arterial puncture often is painful and sometimes is a long drawn out procedure.   We have attempted arterial puncture on experienced subjects standing at attention but usually the subject has fainted before the puncture was completed even in the absence of pain. There must remain therefore some doubt as to whether the differences observed are in all cases real or whether they may be due in part to the conditions of the experiment.

SUMMARY.

In a series of normal subjects at rest and at work and in another series of pathological subjects only small differences were found between arterial and venous blood both in respect to carbonic acid capacity at a partial pressure of 40 mm. of CO_2 and in respect to oxygen capacity.

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THE EFFECT OF IONIZATION UPON OPTICAL ROTATION.

IV. FURTHER STUDIES ON AMINO ACIDS AND PEPTIDES.

By P. A. LEVENE, LAWRENCE W. BASS, ALEXANDRE ROTHEN and
ROBERT E. STEIGER.

(*From the Laboratories of The Rockefeller Institute for Medical Research,
New York.*)

In a series of publications[1] from this laboratory it has been
shown that the change in rotation on passing from an unionized
molecule to the mono-ion, or from the mono-ion to the di-ion, is a
linear function of the degree of dissociation.   The point has been
emphasized that this phenomenon can be employed to determine
dissociation constants lying in ranges in which electrometric
titration ˙does not give accurate results.   Thus, for example, a
dissociation constant $pG' = 13.5$ was found by this method for the
enol form of dextro-alanyl-dextro-alanine anhydride.

The present paper consists of a report of additional experiments
which corroborate the conclusions drawn from earlier results.
By means of a more accurate polarimeter, reading to $0.001°$, we
are now able to obtain more accurate values.   With the data for
levo-leucine (Table III) the agreement is excellent between the
values of pG'_1 and pG'_2 obtained by titration and by rotation.
It must be emphasized, however, that the accuracy for any com-
pound is conditioned by the difference between the rotations of the
two molecular species which are in equilibrium.

Our results also furnish evidence in favor of the view, expressed
in the second paper of this series, that peptides have an enolic
dissociation constant high on the alkaline side.   Our previous
results were limited to a single observation on a sharp change in

[1] Levene, P. A., Simms, H. S., and Bass, L. W., *J. Biol. Chem.*, **70**, 243
(1926).   Levene, P. A., Bass, L. W., Steiger, R. E., and Bencowitz, I., *J.
Biol. Chem.*, **72**, 815 (1927).   Levene, P. A., and Bass, L. W., *J. Biol. Chem.*,
74, 727 (1927).

TABLE I.

Summary of Titration and Rotation Constants. *

	Concentration.	pG'₁	pG'₂	$[M_1]^{25}_D$ Molecular species in acid range.	$[M_0]^{25}_D$ Molecular species in neutral solution.	$[M_2]^{25}_D$ Molecular species in alkaline range.	$[M_1]^{25}_{5461}$ Molecular species in acid range.	$[M_0]^{25}_{5461}$ Molecular species in neutral solution.	$[M_2]^{25}_{5461}$ Molecular species in alkaline solution.
	M			*degrees*	*degrees*	*degrees*	*degrees*	*degrees*	*degrees*
Levo-leucine............	0.100	2.31	9.64	+18.5	−13.1	+11.2			
Levo-valine............	0.500 } 0.250	2.24	9.65				−34.1	−8.3	−21.4
Glycyl-levo-valine......	0.250	3 17	8.25				+26.5	+43.4	+15.6
Glycyl-dextro-valine ...	0.100	3.14	8.20	−25.3	−38.6	−13.7			
Glycyl-dextro-isovaline.	0.100	3.44	8.27	−13.1	+2.3	+15.5			

* Values of pG' are calculated from titration data. Values of $[M_1]$ and $[M_2]$ are read from a plot of b', the corrected equivalents of acid or base, against $[M]$.

the rotation of glycyl-levo-alanyl-levo-alanyl-glycine between pH 12.83 and pH 13.35.   The titration-rotation data of amino acids show that there is some change in the rotation at very high pH and at very low pH; this change in rotation is probably due to the change in the nature of the solvent.   In dipeptides the corresponding change from the rotation of the molecular species existing on the alkaline side is, however, of sufficient magnitude to justify the opinion that there is a third dissociation constant.[2]   No technique has yet been developed which will enable us to evaluate this constant.

<div align="center">TABLE II.</div>
<div align="center">*Analyses of Compounds.*</div>

	Sample No.	Calculated.			Found.			
		C	H	N	C	H	N	Moisture.
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Levo-valine..................	548	51.26	9.47	11.97	50.68	9.45	12.13	0.60
Levo-leucine.................	989	54.92	9.99	10.69	54 71	10.07	10.65	0.53
Glycyl-dextro-valine	2526	48.24	8.10	16.09	48.22	8.22	16.10	1.60
Glycyl-levo-valine	2539	48.24	8.10	16.09	48.07	8.06	16.15	1.30
Glycyl-dextro-isovaline	2528	48.24	8.10	16.09	48.30	7.80	16.00	1.01

<div align="center">EXPERIMENTAL.</div>

<div align="center">*Preparation of Compounds.*</div>

The analyses of the compounds used in these experiments are recorded in Table II.

Levo-valine was prepared by the hydrolysis of the pure levo-formyl-valine obtained by resolving the inactive formyl compound with brucine in methyl alcoholic solution.[3]

Levo-leucine (Kahlbaum) was converted into the hydrochloride which crystallized from the concentrated hydrochloric acid solution.   The amino acid was then liberated in the usual way by ammonium hydroxide.   20 gm. of dry, finely powdered material were dissolved in 400 cc. of boiling water in the presence of some

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[2] The rates of hydrolysis of the peptides under these conditions are too low to account for the change.

[3] Fischer, E., *Ber. chem. Ges.*, **39**, 2322 (1906).

norit; 200 cc. of absolute alcohol were added to the filtrate. The crystals were filtered off after the mixture had been allowed to stand in ice.

Glycyl-Dextro-Valine.[4] *Glycyl-Levo-Valine.*[5] *Glycyl-Dextro-Isovaline.*[6]—The preparation of these compounds has been described in previous publications.

Potentiometric and Polarimetric Data.

The electrometric titrations of all compounds were made at 25° in water-jacketed hydrogen electrode cells. The calculations from the titration data were made by the method described in previous publications,[7] the following equations being employed.

$$ph = pH - \log \tau_H$$
$$poh = (13.89 - pH) - \log \tau_{OH}$$
$$b' = \frac{b - a}{c} + \frac{h - oh}{c}$$

H refers to activities and h and oh to concentrations. The values of pG' were obtained from the relation

$$pG' = pH - \log \frac{\gamma}{1 - \gamma}$$

The symbol γ is used throughout to denote degree of ionization, in order to avoid confusion with α, used for rotation.

The rotations of the levo-valine and glycyl-levo-valine solutions were measured at 25° for the wave-length $\lambda = 5461$ Å. in a Schmidt and Haensch polarimeter reading to 0.01°. The rotations of the levo-leucine, glycyl-dextro-valine, and glycyl-dextro-isovaline solutions were measured at 25° for the wave-length $\lambda = 5892$ Å. in a Schmidt and Haensch polarimeter reading to 0.001°. The values of [M] used in plotting the rotation-ioniza-

[4] Fischer, E , and Scheibler, H , *Ann. Chem.*, **363**, 138, 140 (1908).

[5] Levene, P. A., Bass, L. W., and Steiger, R. E., *J. Biol. Chem.*, **81**, 221 (1929).

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[7] Levene, P. A., Bass, L. W., Steiger, R. E., and Bencowitz, I., *J. Biol. Chem.*, **72**, 815 (1927). See also, Simms, H. S., *J. Am. Chem. Soc.*, **48**, 1239 (1926). The values of log τ_H and log τ_{OH} are taken from Levene, P. A., s, L. W., and Simms, H. S., *J. Biol. Chem.*, **70**, 229 (1926).

tion curves in Fig. 1 are set in bold face in Tables III to VII.
The values of $[M_1]$ and $[M_2]$, the rotations of the molecular species
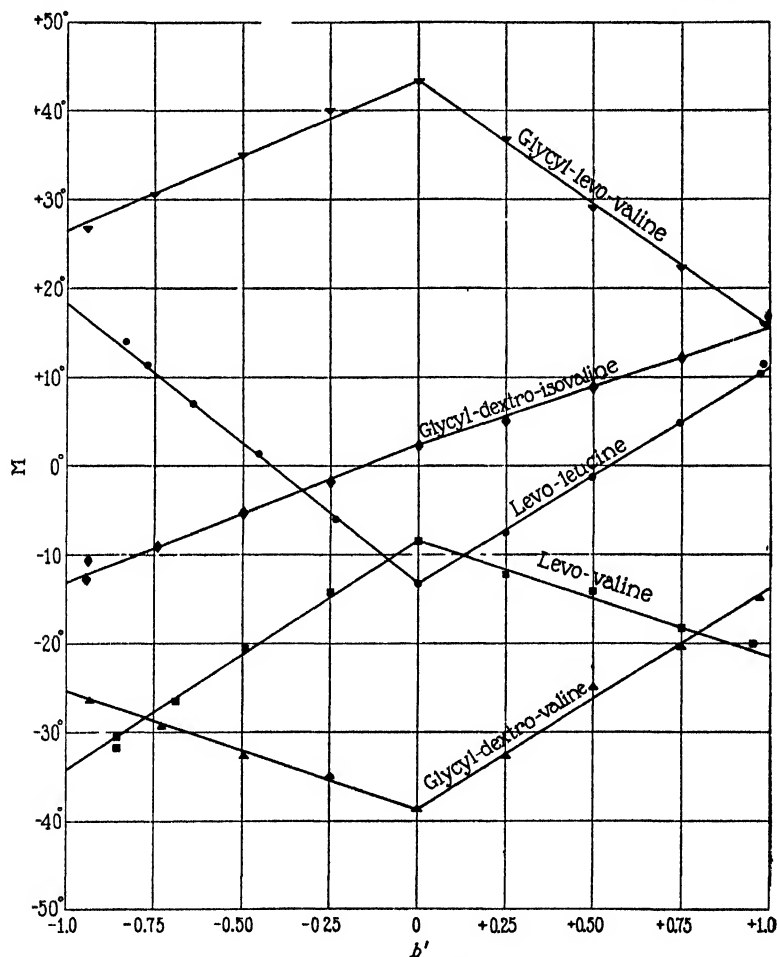
FIG. 1. Molecular rotations plotted against corrected equivalents of
acid or base.

existing in the acid and alkaline ranges respectively, are taken
as the intercepts of these curves at $b' = -1.0$ and $b' = +1.0$. The
values of $[M_1]$, $[M_0]$, and $[M_2]$ are recorded in Table I.

TABLE III.

Levo-Leucine (0.100 Molar). *

$\frac{b-a}{c}$	pH	Titration.					α_D^{25}	$[M]_D^{25}$	Rotation			
		b'	γ_1	γ_2	pG'$_1$	pG'$_2$			γ_1	γ_2	pG'$_1$	pG'$_2$
—40.00	—0.8						+0.776	+19.4$_0$				
—10.00	0.4						+0.699	+17.4$_7$				
— 2.000	1.01						+0.640	+16.0$_0$				
— 1.250	1.46	—0.833	0.167		(2.16)		+0.561	**+14.0$_2$**	0.142		(2.24)	
— 1.000	1.72	—0.771	0.229		(2.25)		+0.459	**+11.4$_7$**	0.221		2.27	
— 0.750	2.03	—0.640	0.360		2.28		+0.284	+ 7.1$_0$	0.361		2.28	
— 0.500	2.40	—0.454	0.546		2.32		+0.061	+ 1.5$_2$	0.538		2.32	
— 0.250	2.84	—0.234	0.766		2.33		—0.237	— 5.9$_2$	0.772		2.31	
0	5.9	0					—0.525	—13.1$_2$				
0.250	9.15	0.250		0.250		9.63	—0.294	— 7.3$_5$		0.239		9.65
0.500	9.65	0.500		0.500		9.65	—0.040	— 1.0$_0$		0.498		9.65
0.750	10.12	0.748		0.748		9.65	+0.202	**+ 5.0$_5$**		0.745		9.66
1.000	11.18	0.976		0.976		(9.57)	+0.419	**+10.4$_7$**		0.972		9.64
1.250	12.21						+0.465	**+11.6$_2$**				
2.000	12.85						+0.454	+11.3$_5$				
10.00	13.6						+0.438	+10.9$_5$				
40.00	14.2						+0.416	+10.4$_0$				
Average					2.31	9.64					2.30	9.65

* Values of pG' in parentheses have not been included in calculating average values; inferior numbers are used to indicate figures which are not significant.   Values of [M] in bold face type have been used in plotting Fig. 1.

Experimental Data.

Levo-Leucine.—Samples of 0.0025 mol plus the required volumes of standard acid or alkali were made up to 25.0 cc. in volumetric flasks. The rotations were measured in 4.00 dm. tubes. The data are recorded in Table III. In Fig. 1 the values of b' are plotted against [M].

From the rotation data the values of γ were calculated by the equations

$$\gamma_1 = \frac{[M_1] - [M]}{[M_1] - [M_0]} \qquad \gamma_2 = \frac{[M_0] - [M]}{[M_0] - [M_2]}$$

TABLE IV.

Levo-Valine. (*Series A, 0.500 Molar; Series B, 0.250 Molar.*)

Series.	$\dfrac{b-a}{c}$	pH	pG′$_1$	pG′$_2$	α^{25}_{5461}	[M]$^{25}_{5461}$
B	−1.500	0.85			−1.56	−31.2
A	−1.000	1.13			−3.16	−31.6
"	−1.000	1.13			−3.05	−30.5
B	−0.750	1.86	2.21		−1.32	−26.4
A	−0.500	2.26	2.24		−2.05	−20.5
"	−0.250	2.77	2.28		−1.40	−14.0
"	0	6.2			−0.83	− 8.3
"	0.250	9.16		9.64	−1.20	−12.0
"	0.500	9.64		9.64	−1.40	−14.0
B	0.750	10.16		9.68	−0.91	−18.2
A	1.000	12.15			−2.00	−20.0
B	1.500	12.94			−1.04	−20.8
Average...................			2.24	9.65		

From these values of γ_1 and γ_2 the values of pG′ were calculated by the equation

$$pG' = pH - \log \frac{\gamma}{1 - \gamma}$$

Levo-Valine.—Two series of experiments were run. The data are recorded in Table IV; b' is plotted against [M] in Fig. 1.

Series A.—Samples of 0.0050 mol plus the required volumes of standard acid or alkali were made up to 10.0 cc. in volumetric flasks. The rotations were measured in 2.00 dm. tubes.

TABLE V.
Glycyl-Levo-Valine (0.250 Molar).

$\frac{b-a}{c}$	pH	pG'$_1$	pG'$_2$	α^{25}_{5461}	$[M]^{25}_{5461}$
−2.000	0.66			+1.23	+24.6
−1.500	0.90			+1.22	+24.4
−1.000	1.82			+1.34	+26.8
−0.750	2.68	3.16		+1.53	+30.6
−0.500	3.17	3.17		+1.75	+35.0
−0.250	3.66	3.18		+2.01	+40.2
0	5.8			+2.17	+43.4
0.250	7.76		8.24	+1.85	+37.0
0.500	8.24		8.24	+1.46	+29.2
0.750	8.75		8.27	+1.12	+22.4
1.000	10.12			+0.80	+16.0
1.500	12.92			+0.87	+17.4
2.000	13.15			+0.89	+17.8
Average................		3.17	8.25		

TABLE VI
Glycyl-Dextro-Valine (0.100 Molar).

$\frac{b-a}{c}$	pH	pG'$_1$	pG'$_2$	α^{25}_{D}	$[M]^{25}_{D}$
−40.00	−0.8			−0.425	−21.2$_5$
−10.00	0.4			−0.505	−25.2$_5$
− 1.250	1.57			−0.529	−26.4$_5$
− 0.750	2.71	3.13		−0.585	−29.2$_5$
− 0.500	3.16	3.15		−0.652	−32.6$_0$
− 0.250	3.63	3.14		−0.699	−34.9$_5$
0	5.7			−0.722	−38.6$_0$
0.250	7.75		8.22	−0.651	−32.5$_5$
0.500	8.21		8.21	−0.496	−24.8$_0$
0.750	8.66		8.18	−0.405	−20.2$_5$
1.250	12.23			−0.293	−14.6$_5$
10.00	13.6			−0.363	−18.1$_5$
40.00	14.2			−0.373	−18.6$_5$
Average................		3.14	8.20		

Series B.—Samples of 0.0025 mol plus the required volumes of standard acid or alkali were made up to 10.0 cc. in volumetric flasks.   The rotations were measured in 2.00 dm. tubes.

Glycyl-Levo-Valine.—Samples of 0.0025 mol plus the required volumes of standard acid or alkali were made up to 10.0 cc. in volumetric flasks. The rotations were measured in 2.00 dm. tubes. The data are recorded in Table V; b' is plotted against [M] in Fig. 1.

Glycyl-Dextro-Valine.—Samples of 0.0010 mol plus the required volumes of standard acid or alkali were made up to 10.0 cc. in

TABLE VII.

Glycyl-Dextro-Isovaline (0.100 Molar).

$\frac{b-a}{c}$	pH	pG'$_1$	pG'$_2$	α_D^{25}	$[M]_D^{25}$
−40 00	−0.8			−0.228	−11.4$_0$
−10 00	0.4			−0.213	−10.6$_5$
− 2 000	1.03			−0.221	−11.0$_5$
− 1.250	1.59			−0.252	−12.6$_0$
− 1 000	2 28	3.43		−0.210	−10.5$_0$
− 0 750	2 98	3.43		−0.181	− 9.0$_5$
− 0 500	3 46	3.45		−0 106	− 5.3$_0$
− 0 250	3.93	3.45		−0.036	− 1.8$_0$
0	5.6			+0.047	+. 2.3$_5$
0 250	7.78		8.26	+0.105	+ 5.2$_5$
0 500	8.29 ˙		8.29	+0.179	+ 8.9$_5$
0 750	8.73		8.25	+0.244	+12.2$_0$
1.000	10 54			+0.334	+16.7$_0$
1.250	12.20			+0.315	+15.7$_5$
2 000	12.90			+0.295	+14.7$_5$
10 00	13.6			+0.264	+13.2$_0$
40.00	14.2			+0.114	+ 5.7$_0$
Average		3.44	8.27		

volumetric flasks. The rotations were measured in 2.00 dm. tubes. The data are recorded in Table VI; b' is plotted against [M] in Fig. 1.

Glycyl-Dextro-Isovaline.—Samples of 0.0010 mol plus the required volumes of standard acid or alkali were made up to 10.0 cc. in volumetric flasks. The rotations were measured in 2.00 dm. tubes. The data are recorded in Table VII: b' is plotted against [M] in Fig. 1.

THE RELATION OF CHEMICAL STRUCTURE TO THE RATE OF HYDROLYSIS OF KETOPIPERAZINES.

I. HYDROLYSIS OF N-METHYLKETOPIPERAZINES BY ALKALI.

BY P. A. LEVENE, LAWRENCE W. BASS, AND ROBERT E. STEIGER.

(*From the Laboratories of The Rockefeller Institute for Medical Research, New York.*)

The experiments to be described here were initiated with the object of obtaining data required for the interpretation of observations on the racemization of proteins.[1] The action of alkali on proteins differs from the action of alkali on the simple peptides and simple ketopiperazines which have thus far been examined.[2] Simple peptides, on long standing at 20–25° with 0.1 N, 0.2 N, 0.5 N, or 1.0 N alkali, undergo comparatively little racemization. Simple ketopiperazines, on standing with 0.1 N alkali, undergo rapid racemization.   With 1.0 N alkali, however, they are rapidly hydrolyzed to the corresponding dipeptides, which suffer little racemization; hence the racemization of ketopiperazines under these conditions is much lower.

The proteins which have been studied show the following difference from the behavior of these simpler compounds.   Proteins show a considerable degree of racemization under the influence of weak alkali in a comparatively short time (24 hours); in this respect they resemble ketopiperazines.   On the other hand, the racemization is even higher on treatment with strong alkali.

The reasons for the difference in the behavior of proteins are not as yet clear.   It may be due to the presence in proteins of

[1] Levene, P. A., and Bass, L. W., *J. Biol. Chem.*, **74**, 715 (1927); **78**, 145 (1928).

[2] Levene, P. A., and Pfaltz, M. H., *J. Biol. Chem.*, **63**, 661 (1925); *J. Gen. Physiol.*, **8**, 183 (1925); *J. Biol. Chem.*, **68**, 277 (1926); **70**, 219 (1926). Levene, P. A., and Steiger, R. E., *J. Biol. Chem.*, **76**, 299 (1928).

ketopiperazines more stable than those which have thus far been studied, or it may be due to the presence of peptides with higher rates of racemization than those reported in previous publications. In order to explain the conduct of proteins it is therefore necessary to extend our information, on one hand, on the stability of ketopiperazines and, on the other, on the rates of racemization of peptides.

TABLE I.

Rates of Hydrolysis of Ketopiperazines by Sodium Hydroxide. *

Glycyl-glycine anhydride.†			Glycyl-sarcosine anhydride.			Sarcosyl-sarcosine anhydride.			d,l-N-Methyl-alanyl-sarcosine anhydride.			d,l-N-Methyl-valyl-sarcosine anhydride.‡		
t	V	$k \cdot 10^3$	t	V	$k \cdot 10^3$	t	V	$k \cdot 10^3$	t	V	$k \cdot 10^3$	t	V	$k \cdot 10^3$
min.	*cc.*		*min.*	*cc.*		*min.*	*cc.*		*min.*	*cc.*		*hrs.*	*cc.*	
1	1.04	(233)	1	0.58	114	1	0.61	122	5	0.47	(18)	6.5	0.11	0.05
2	1.47	(192)	2	0.99	109	2	1.04	117	10	0.68	(13)	10	0.19	0.06
3	1.79	(180)	4	1.61	112	3	1.44	124	40	1.42	9	48	0.81	0.06
4	1.94	163	5	1.76	106	4	1.67	120	60	1.67	8	96	1.33	0.06
5	2.10	159	8	2.15	106	6	2.02	119	90	1.99	8	120	1.50	0.06
6	2.23	161	10	2.24	98	8	2.16	108	120	2.10	7	144	1.59	0.05
8	2.36	156	12	2.33	97	10	2.26	102	140	2.18	7			
10	2.44	162	16	2.44	101	12	2.35	102	240	2.25	6			
60	2.52		60	2.47		60	2.48		1440	2.41				
Average		160			105			114			8			0.06

* Values of $k \cdot 10^3$ in parentheses have not been used in calculating average values.

† The large deviations from the average of the values of $k \cdot 10^3$ for 1, 2 and 3 minutes are probably due to errors in the time intervals. The hydrolysis proceeds so rapidly that the time required to pipette the samples introduces a large error.

‡ The time is given in hours; $k \cdot 10^3$ is calculated for minutes.

The present paper is the first of a series dealing with the stability of ketopiperazines differing in structure. The experimental results are presented in Table I. From these data it is seen that glycyl-glycine, glycyl-sarcosine, and sarcosyl-sarcosine anhydrides are split very rapidly at a pH of about 13.4; glycyl-glycine anhydride is split most rapidly, while the other two anhydrides are split at a lower rate. The effect of substitution of an alkyl rest

on the α-carbon is marked. Thus N-methylalanyl-sarcosine anhydride, differing from sarcosyl-sarcosine anhydride only in that it contains a methyl group attached to one α-carbon, hydrolyzes under the same conditions at a much lower rate. The effect is still more pronounced in the case of N-methylvalyl-sarcosine anhydride.

These results agree in a general way with the rates of hydrolysis of ketopiperazines calculated from data on the hydrolysis of the corresponding peptides in acid solution.[3] Better agreement could not be expected in view of the high concentration of alkali used in the present experiments.

EXPERIMENTAL.

Preparation of Ketopiperazines.

The analyses of the ketopiperazines used in these experiments are summarized in Table II.

Glycyl-glycine anhydride was prepared from glycine ethyl ester[4] by the method of Fischer and Fourneau.[5]

Glycyl-sarcosine Anhydride.—A suspension of 24 gm. of glycyl-sarcosine[6] in 240 cc. of dry methyl alcohol was saturated with dry hydrogen chloride gas under moderate cooling. The solution was evaporated to dryness under reduced pressure. A second esteri-

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[3] Levene, P. A., Simms, H. S., and Pfaltz, M. H., *J. Biol. Chem.*, **61**, 445 (1924).

[4] Glycine ethyl ester is most conveniently prepared from glycine ethyl ester hydrochloride by a method outlined by Foreman (Foreman, F. W., *Biochem. J.*, **13**, 382 (1919)). A mixture of 560 gm. (4 mols) of crude glycine ethyl ester hydrochloride (obtained from silk), 1400 cc. of dry chloroform, and 500 gm. (theory: 343 gm. = 2 mols) of anhydrous barium hydroxide was shaken in a 5 liter Pyrex round bottom flask, the reaction mixture being cooled by running tap water. After the reaction had slowed down, the mixture was shaken 2½ hours in a shaking machine. When the mixture was allowed to stand, the $BaCl_2 \cdot 2H_2O$ and excess of barium hydroxide settled immediately. The precipitate was filtered off and washed thoroughly with dry chloroform. The chloroform solution of glycine ethyl ester was then concentrated under reduced pressure and finally was carefully fractionated by means of a good column. The glycine ethyl ester distilled at the end, boiling at 61° under 20 mm. The yield was 320 gm. (80 per cent of the theory).

[5] Fischer, E., and Fourneau, E., *Ber. chem. Ges.*, **34**, 2870 (1901).

[6] Levene, P. A., Simms, H. S., and Pfaltz, M. H., *J. Biol. Chem.*, **61**, 450 (1924).

fication identical to the first was then performed.   The residue
left after evaporation was dissolved in 50 cc. of dry methyl alcohol.
240 cc. of a methyl alcoholic ammonia solution saturated at 0°
were added and the solution was then saturated under cooling in
ice with dry ammonia gas.   It was allowed to stand overnight in
ice and was then evaporated to dryness under reduced pressure.
Benzene was repeatedly added to the residue and each time dis-
tilled off under reduced pressure.   The dry residue, consisting
of anhydride and ammonium chloride, was extracted with hot
absolute alcohol.   The alcoholic filtrate from the ammonium
chloride was evaporated to dryness under reduced pressure and

TABLE II.

Analyses of Ketopiperazines.

Ketopiperazine.	Sample No.	Calculated.			Found.			
		C	H	N	C	H	N	Moisture.
		per cent	per cent	per cent	per cent	per cent	per cent	per cent
Glycyl-glycine anhydride...	2696	42.08	5.30	24.57	41.83	5.23	23.97	0.60
Glycyl-sarcosine "	2722	46.85	6.29	21.88	46.96	5.78	21.63	0.13
Sarcosyl-sarcosine "	2723	50.67	7.09	19.72	50.58	6.91	19.70	1.51
d,l-N-Methylalanyl-sarcosine anhydride.....................	2724	53.81	7.75	17.94	53.77	7.76	17.57	1.09
d,l-N-Methylvalyl-sarcosine anhydride...................	2725	58.65	8.76	15.21	58.44	8.32	14.75	2.43

the residue was repeatedly extracted with benzene at boiling tem-
perature.   The benzene solutions were cooled in ice; the crystals
deposited were washed on the filter with benzene.   The com-
pound melted at 142–143° (corrected).

4.340 mg. substance: 7.475 mg. CO_2 and 2.245 mg. H_2O.
0.1000 gm.   "   : 15.45 cc. 0.1 N HCl (Kjeldahl).
$C_5H_8O_2N_2$ (128.08).   Calculated.   C 46.85, H 6.29, N 21.88.
                                Found.   " 46.96, " 5.78, " 21.63.
Moisture 0.13 per cent.

Sarcosyl-sarcosine anhydride[7] was prepared by fusing sarcosine
at 220° under reduced pressure.   It was purified by crystalliza-
tion from absolute alcohol.

[7] *Cf.* Mylius, F., *Ber. chem. Ges.*, **17,** 287 (1884).

d,l-N-Methylalanyl-sarcosine anhydride was prepared according to the directions of Levene, Simms, and Pfaltz.[8]

d,l-N-Methylvalyl-sarcosine anhydride was prepared according to the directions of Levene, Simms, and Pfaltz.[9]

Procedure in Hydrolysis Experiments.

The following procedure was used for each ketopiperazine. The rates of hydrolysis were measured at 25°.

A sample of 0.005 mol (corrected for moisture) of ketopiperazine was weighed in a 50.0 cc. volumetric flask. The sample was dissolved in water, 25.0 cc. (5 equivalents) of 1.0 N NaOH were added, the volume was made up to 50.0 cc., and the solution was thoroughly mixed. These operations were performed as rapidly as possible. At definite time intervals 5.00 cc. samples were withdrawn by means of pipettes; these samples were emptied into neutralizing solutions as described below.

Before beginning an experiment, a series of neutralizing solutions was prepared; each solution consisted of 10 cc. of alcohol, 2.50 cc. of 1.0 N hydrochloric acid, and 2 drops of a 1 per cent solution of alizarin yellow.[10] As soon as a sample had been removed from the reaction flask, it was emptied into one of these solutions. A solution of 0.2 N sodium hydroxide was then added from a small bore burette to the first definite red color. For glycyl-glycine, glycyl-sarcosine, and sarcosyl-sarcosine anhydrides, which hydrolyze very rapidly, the samples were allowed to run into the neutralizing solutions at such a rate as to half empty the pipettes at the time intervals shown in the data.

The rates of hydrolysis were calculated by the equation

$$kt = \log_{10} \frac{a}{-\ -}$$

The calculations were made on the assumption that no hydrolysis of the peptides to the simple amino acids occurred; this secondary

[8] Levene, P. A., Simms, H. S., and Pfaltz, M. H., *J. Biol. Chem.*, **70**, 262 (1926).

[9] Levene, P. A., Simms, H. S., and Pfaltz, M. H., *J. Biol. Chem.*, **70**, 263 (1926).

[10] The application of alizarin yellow to the titration of amino acids and peptides has been described by Felix, F., and Müller, H., *Z. physiol. Chem.*, **171**, 4 (1927).

hydrolysis takes place so slowly under these conditions that it may be disregarded.   At least two parallel experiments were performed with each anhydride.   The data are summarized in Table I.   It should be noted that in the case of the unsymmetric anhydrides the constants given represent the resultant of the rates of formation of the two possible isomeric dipeptides.

The rates of hydrolysis of glycyl-glycine, glycyl-sarcosine, and sarcosyl-sarcosine anhydrides are so rapid that a slight error in the time interval introduces a considerable error in the constant. The agreement in our data is as good as can be expected under the conditions which we have used.   Furthermore, the change in the pH of the hydrolysis solutions, due to the buffering action of the peptide formed, has an influence on the reaction constants.[11] By the use of a large excess of alkali, this change in pH during the reaction was reduced considerably.

[11] von Euler, H., and Pettersson, E., *Z. physiol. Chem.*, **158**, 7 (1926). Abderhalden, E., and Kröner, W., *Fermentforschung*, **10**, 25 (1928).

ON THE CONFIGURATIONAL RELATIONSHIP OF LACTIC ACID AND 2-CHLOROPROPIONIC ACID.

WITH A FURTHER NOTE ON THE CONFIGURATIONAL RELATIONSHIP OF LACTIC ACID AND METHYLPROPYL CARBINOL.

By P. A. LEVENE and H. L. HALLER.

(From the Laboratories of The Rockefeller Institute for Medical Research, New York.)

(Received for publication, January 26, 1929.)

The considerations which led to the investigation, the results of which are reported in this communication, have been discussed in the previous communication of this series.[1] As was there stated, the fundamental assumption is that the replacement of a hydroxyl in an optically active secondary alcohol by a halogen is not associated with a Walden inversion. If the correctness of this assumption is granted, it is possible to correlate the configurations of lactic and chloropropionic acids by the following set of reactions:

$$
\begin{array}{ccccc}
& CH_3 & CH_3 & CH_3 & \\
& | & | & | & \\
& CH_2 & CH & CH & \\
& | & \| & \| & \\
COOH & CH_2 & CH & CH & COOH \\
\\
HOCH & HOCH & HOCH & ClCH & ClCH \\
| & | & | & | & | \\
CH_3 & CH_3 & CH_3 & CH_3 & CH_3 \\
\text{Dextro.} & \text{Dextro.} & \text{Levo.} & \text{Levo.} & \text{Dextro.} \\
\text{I.} & \text{II.} & \text{III.} & \text{IV.} & \text{V.}
\end{array}
$$
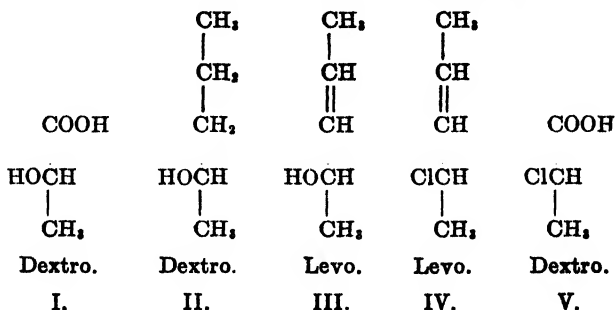
Thus, on the basis of this set of reactions, dextrorotatory lactic acid is configurationally related to dextro-chloropropionic acid. The same conclusion had been reached previously by Levene and Mikeska[2] on the basis of a totally different and independent assumption. This

[1] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **81**, 425 (1929).
[2] Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, **70**, 365 (1926).

fact naturally adds strength to the view on the configurational relationship accepted by us, although it may be contended, and with justice, that for the present the possibility is not entirely excluded that both our assumptions need to be reversed in their sense. Whatever the ultimate outcome of the controversy may be, the method presented here gives a direct way of correlating the configurations of all α-halogeno acids to one reference substance; namely, to the halogenopropionic acid.

Again in this case we took occasion to test the configuration of methylpropyl carbinol with reference to lactic acid.[3] The set of reactions chosen for this purpose is the following:

```
                                    COOH
                                     |
                                 ,HOCH
                                /    |
                               /    CH₃
          CH₃                 /    Dextro.
           |                 /
          CH                /      VII.
          ‖                /
          CH ─────────────
           |              \        CH₃
         HOCH              \        |
           |               \      CH₂
          CH₃               \      |
        Levo.                \    CH₂
                              \    |
         VI.                   ➘  HOCH
                                   |
                                  CH₃
                                Dextro.

                                 VIII.
```
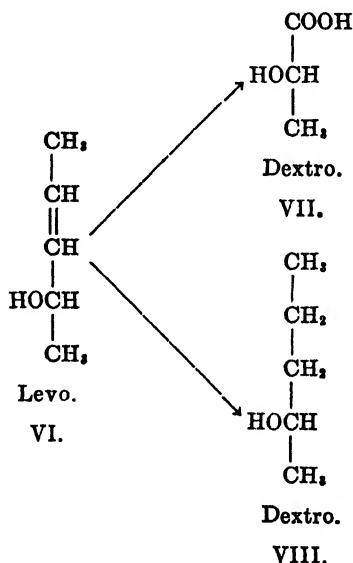
Thus the conclusion reached previously has been confirmed by this second method.

EXPERIMENTAL.

Δ^2-*Pentenol*-(*4*).—The inactive carbinol was obtained by condensation of methyl magnesium iodide with crotonic aldehyde in

[3] Levene, P. A., Haller, H. L., and Walti, A., *J. Biol. Chem.*, **72**, 591 (1927).

the usual manner.   The carbinol distilled at 119–122° at atmospheric pressure.

Resolution of Δ²-*Pentenol-(4).*—The acid phthalic ester was prepared by heating a solution of 86 gm. of the carbinol and 148 gm. of phthalic anhydride in 175 cc. of dry pyridine on the steam bath for 1 hour.   The isolation and purification of the acid phthalate were carried out in the usual manner.[4]   It melted at 90°.

A warm solution of 50 gm. of pentenol acid phthalate in 400 cc. of acetone was neutralized with 100 gm. of brucine.   The solution was heated to boiling and filtered.   On cooling, the brucine salt crystallized.   After it had been recrystallized several times from acetone, the brucine salt had the following rotation in absolute alcohol.

$$[\alpha]_D^{24} = \frac{-0.77° \times 100}{1 \times 5.6} = -13.7°.$$

The brucine salt was decomposed with dilute hydrochloric acid and the acid phthalate was extracted with ether; the ether extract was washed with water, dried over anhydrous sodium sulfate, and the ether was then removed.   The acid phthalate readily crystallized.   In ether the rotation was

$$[\alpha]_D^{24} = \frac{+8\ 00° \times 100}{1 \times 26} = +30\ 8°.$$

The acid phthalate ($[\alpha]_D^{24} = +30.8°$ in ether), obtained as described above, was dissolved in an aqueous solution of sodium hydroxide (3 mols) and the solution was steam-distilled.   The carbinol was extracted with ether.   The ether extract was dried over anhydrous potassium carbonate.   After removal of the ether the carbinol was distilled under reduced pressure, the receiver being cooled in a CO_2-ether bath.   It was then redistilled at atmospheric pressure.   It boiled at 120–122° at atmospheric pressure.   It analyzed as follows:

5.350 mg. substance: 13.700 mg. CO_2 and 5.580 mg. H_2O.
$C_5H_{10}O$.   Calculated.   C 69.70, H 11.62.
           Found.   " 69.83, " 11.67.

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[4] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **79**, 475 (1928).

In ether the rotation was

No. 1167. $[\alpha]_D^{25} = \dfrac{-\,0.92° \times 100}{1 \times 24.8} = -\,3.7°.$

An aliquot part of the above solution was diluted with an equal volume of ether.   The rotation then was

$$[\alpha]_D^{25} = \dfrac{-\,0.76° \times 100}{1 \times 12.4} = -\,6.1°.$$

α-Naphthylurethane of Levo-Δ²-Pentenol-(4).—1 gm. of α-naphthyl isocyanate was added to 0.5 gm. of levo-Δ²-pentenol-(4) (No. 1167).   The reaction mixture was heated on the steam bath for 10 minutes and then allowed to stand overnight.   The product was extracted with hot absolute alcohol, filtered, and placed in the ice box.   The urethane readily crystallized (No. 1180).   It was filtered off and to the filtrate water was added.   A second crop of crystals soon formed (No. 1192).   After drying, they analyzed as follows:

No. 1180.   0.1000 gm. substance: (Kjeldahl) 3.83 cc. 0.1 N HCl.
"   1192.   0.1002 "   "   :   "   3.80 " 0.1 " "
          $C_{16}H_{17}O_2N$. Calculated.   N 5.49.
          No. 1180.   Found.   " 5.36.
          "   1192.   " 5.31.

No. 1180 melted at 130° and No. 1192 melted at 109–113°.   The rotation of No. 1180 in absolute alcohol was

$$[\alpha]_D^{25} = \dfrac{+\,0.45° \times 100}{2 \times 2.45} = +\,9.2°.$$

The rotation of No. 1192 in absolute alcohol was

$$[\alpha]_D^{25} = \dfrac{+\,0.25° \times 100}{2 \times 2.06} = +\,6.1°.$$

Levo-4-Chloropentene-(2).—A solution of 25 gm. of Δ²-pentenol-(4) (No. 1167) in 5 cc. of pyridine was slowly dropped into 15 gm. of phosphorus trichloride which was well cooled in an ice water bath.   The reaction mixture was constantly shaken during the addition of the carbinol.   It was then allowed to stand at room

temperature for 1 hour. The chloride was then distilled under reduced pressure (p = about 20 mm.) the receiver being cooled in a CO_2-ether bath. The chloride analyzed as follows:

0.0882 gm. substance: 0.1225 gm. AgCl.
    No. 1169. C_5H_9Cl. Calculated. Cl 33.9. Found. Cl 34.36.

In a 1 dm. tube without solvent $\alpha_D^{23} = -8.25°$. In ether the rotation was

$$[\alpha]_D^{23} = \frac{-1.45° \times 100}{1 \times 16.0} = -9.1°.$$

In absolute alcohol the rotation was

$$[\alpha]_D^{23} = \frac{-1.10° \times 100}{1 \times 12.5} = -8.8°.$$

Dextro-2-Chloropropionic Acid.—The 4-chloropentene-(2) (No. 1169) obtained as described above was ozonized in chloroform solution in 5 gm. lots. The procedures for ozonization, decomposition, and oxidation were the same as those described in the ozonization of 4-chloropentene-(1) to obtain 3-chlorobutyric acid.[1] The chloropropionic acid was fractionally distilled and a fraction boiling at 60–64°, p = 0.25 − 0.5 mm., was collected. It analyzed as follows:

0.1186 gm substance: 0.1582 gm. AgCl.
      $C_3H_5O_2Cl$. Calculated. Cl 32.72. Found. Cl 33.00.
0.2080 gm. substance: 18.6 cc. 0.1 N NaOH.
Theory for $C_3H_5O_2Cl$: 19.17" 0.1 "   "

Without solvent the rotation was $\alpha_D^{25} = +2.15°$ (l=1). In ether the rotation was

$$[\alpha]_D^{25} = \frac{+1.10° \times 100}{2 \times 16.7} = +3.3°.$$

0.600 gm. of the acid was dissolved in 5.0 cc. of water. The rotation of this solution was

$$[\alpha]_D^{25} = \frac{+0.48° \times 100}{2 \times 12} = +2.0°.$$

The rotation of the sodium salt was obtained in the following manner. 1.566 gm. of the acid were dissolved in 3.7 cc. of water and to this solution were added 14.4 cc. of 0.1 N NaOH. The rotation was

$$[\alpha]_D^{28} = \frac{-0.20° \times 100}{4 \times 10.32} = -0.48°.$$

Dextro-Methylpropyl Carbinol (Pentanol-(2)).—Levo-Δ^2-pentenol-(4) (No. 1167) was reduced in ether solution with hydrogen in the presence of colloidal palladium as catalyst. The reduction and isolation proceeded in the usual manner.[1] The carbinol distilled at 117–119°. It analyzed as follows:

5.255 mg. substance: 13.085 mg. CO_2 and 6.365 mg. H_2O.
$C_5H_{12}O$. Calculated. C 68.10, H 13.72.
Found. " 67.90, " 13.55.

The rotation in ether was

$$[\alpha]_D^{24} = \frac{+1.56° \times 100}{1 \times 11.6} = +13.5°.$$

In a 1 dm. tube without solvent $\alpha_D^{24} = +7.95°$. It was further identified by conversion into its α-naphthylurethane, which melted at 86–88° and analyzed as follows:

0.1012 gm. substance: (Kjeldahl) 3.95 cc. 0.1 N HCl.
$C_{16}H_{19}O_2N$. Calculated. N 5.44.
Found. " 5.46.

In absolute alcohol the rotation was

$$[\alpha]_D^{28} = \frac{+1.15° \times 100}{2 \times 4.3} = +13.4°.$$

Dextro-2-Hydroxypropionic Acid (Lactic Acid).—Into a solution of 8 gm. of levo-Δ^2-pentenol-(4) ($[\alpha]_D^{23} = \dfrac{-0.30° \times 100}{1 \times 12} = -2.5°$),

in 40 cc. of chloroform, a stream of ozonized oxygen was passed until the solution no longer decolorized bromine in glacial acetic acid. The remaining chloroform was distilled off under reduced pressure and the ozonide poured into water. The solution was

thoroughly shaken, and warmed gently on the steam bath for 15 minutes with constant shaking. An excess of silver oxide was added and the mixture was heated under a reflux condenser on the steam bath for 2 hours. The hot solution was filtered off. The silver was removed with hydrogen sulfide and the free acid was converted into the zinc salt. After drying on a high vacuum pump and finally at 115°, the zinc salt gave the following analysis.

0.3100 gm. substance: 0.08347 gm. ZnO.
$C_6H_{10}O_6Zn$. Calculated. Zn 26.86. Found. Zn 26.9.

In water the rotation of the zinc salt was

$$[\alpha]_D^{25} = \frac{-0.053° \times 100}{4 \times 4} = -0.33°.$$

GUANINEDESOXYPENTOSIDE FROM THYMUS NUCLEIC ACID.

By P. A. LEVENE and E. S. LONDON.

(From the Laboratories of The Rockefeller Institute for Medical Research, New York.)

This article contains a brief report on the isolation of a guanine nucleoside from thymus nucleic acid. The hydrolysis of the nucleic acid which led to the isolation of the substance was accomplished by means of intestinal juice obtained from dogs.

The product of digestion of the nucleic acid was treated with 4 volumes of 95 per cent alcohol and the filtrate concentrated to about 150 cc. On standing, a gelatinous precipitate formed. The microscopic appearance of the substance was very much the same as that of impure guanosine.

The purification of this material has been accomplished by the lead process which had been applied for the purification of other nucleosides.

The pure nucleoside crystallized in long needles, and sometimes in platelets. The analytical data agree with the theory of a guaninedesoxypentoside. In harmony with this assumption is the fact that with Kiliani's reagent the substance gives a greenish blue coloration which on standing assumes a purplish tinge resembling in color the usual biuret test for proteins. The substance in a sealed capillary tube contracts at 200° and does not melt at 290°.

The nucleoside analyzed as follows:

5.730 mg. substance: 9.420 mg. CO_2 and 2.585 mg. H_2O.
4.575 "   "   : 1.034 cc. N_2 at 26° and p = 764.5 mm.
$C_{10}H_{13}N_5O_4$.   Calculated.   C 44.92, H 4.90, N 26.20.
            Found.   " 44.83, " 5.00, " 25.96.

711

The rotation of the substance was

$$[\alpha]_D^{20} = \frac{-0.75° \times 100}{2 \times 1} = -37.5°$$

Hydrolysis of the Nucleoside.

The nucleoside is very unstable: 0.700 gm. of the substance in 20 cc. of $\frac{1}{40}$ N sulfuric acid was completely hydrolyzed in 10 minutes. The base was separated in the same manner as in the case of guanosine. The sulfate of the base analyzed as follows:

4.895 mg. substance: 4.915 mg. CO_2 and 1.645 mg. H_2O.
$(C_5H_5N_5O)_2H_2SO_4 + 2H_2O$.   Calculated.   C 27.50, H 3.66.
                           Found.   " 27.38, " 3.76.

From the filtrate of the base the sugar was obtained in the same manner as in the case of the ribose nucleoside. On standing in a desiccator over P_2O_5 under reduced pressure it crystallized into a solid mass. Without recrystallization, the substance contracted at 60°, melted into an opalescent mass at 83°, which completely clarified at 153°. With Kiliani's reagent the sugar gave the same color test as the nucleoside. A pine stick impregnated with the solution of the sugar turned bluish purple in the vapors of a hydrogen chloride solution. The sugar did not form an osazone.

The sugar analyzed as follows:

6.893 mg. substance: 11.310 mg. CO_2 and 4.580 mg. H_2O.
$C_5H_{10}O_4$.   Calculated.   C 44.75, H 7.51.
             Found.   " 44 74, " 7.43.

THE COLORIMETRIC DETERMINATION OF TOTAL AND INORGANIC SULFATES IN BLOOD SERUM, URINE, AND OTHER BODY FLUIDS.*

By E. G. WAKEFIELD.†

(*From the Division of Medicine, The Mayo Foundation, Rochester, Minnesota.*)

Sulfates are usually determined by precipitation with barium. When the quantity of the precipitate was too small to be determined gravimetrically, it was estimated by comparing the turbidity of the unknown with that of a known solution of barium sulfate (3). A recent modification of the turbidimetric method has been suggested by Denis and Reed (4). They prepared suspensions of the barium sulfate and compared these in a nephelometer, with known solutions. According to De Boer (2), Hamburger precipitated the sulfates with barium and measured the volume of barium sulfate after it had been centrifuged in a specially calibrated tube.

Precipitation of sulfates with benzidine and titration of the dissolved benzidine sulfate with an alkali has long been an accepted method for determining sulfates. Different volumetric procedures in which benzidine is used as the precipitating agent have been described by Fiske (5) and White (11).

Three colorimetric methods for determining sulfates, which depend on the production of color from the benzidine in the precipitated benzidine sulfate, have been suggested. Yoshimatsu (12) developed a brown color by treating solutions of benzidine sulfate with a mixture of potassium iodide, iodine, and ammonia. The brown color thus developed is compared with a known standard. Kahn and Leiboff (9) have diazotized benzidine sulfate and mixed it with phenol in an alkaline medium to produce a yellow color

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* Work done under the direction of Norman M. Keith, Division of Medicine, The Mayo Clinic.

† Fellow in Medicine, The Mayo Foundation.

which is proportional to the amount of benzidine present.   They have used this method to determine inorganic sulfates in urine. Hubbard (7) treated solutions of benzidine sulfate with diluted hydrogen peroxide and ferric chloride.   This produced yellow solutions which were compared with known standards.

It was the principle of the colorimetric method suggested by Hubbard (7) which was used in the development of the following procedure.   Hubbard's report was preliminary and was concerned solely with the determination of inorganic sulfates in blood serum. The reagents originally used by him have been retained.   To obtain uniform results it has been necessary to modify portions of the manipulative procedures and the strengths of some of the solutions.   With these modifications it has been possible to extend this method to permit the determination of the total sulfates of serum and the total and inorganic sulfates in the urine.   It has also been possible to determine total and inorganic sulfates in spinal fluid and in fluids which, in conditions accompanied by edema, collect in the thorax, peritoneal cavity, and other regions.

Controls.

In the beginning, it was necessary to ascertain whether the colors produced by a known amount of benzidine chloride were uniform and proportional to the amount of benzidine present. With the concentrations of acid, ferric chloride, and hydrogen peroxide which were used, reasonably stable colors were produced which could be matched with a satisfactory degree of accuracy.   Readings could be made over a range of the benzidine chloride equivalent to sulfates from 0.15 to 0.015 mg.   The most satisfactory range of color was about the sulfate equivalent of 0.06 mg.

It is essential that glassware be scrupulously clean and free from sulfates.   The glassware is cleaned in the ordinary potassium dichromate-sulfuric acid cleaning solution.   All traces of the cleaning solution are removed by washing ten or fifteen times in distilled water and finally in sulfate-free water.

The reagents must be pure and free from sulfates.   Commercial trichloroacetic acid contains sulfates and must be purified.   This is done by distillation under reduced pressure.   To the molten trichloroacetic acid are added 5 cc. of a 0.5 per cent solution of

benzidine base for each 100 cc. of the acid. The benzidine holds any sulfates, and the distillate under these conditions is usually sulfate-free and benzidine-free. Sulfate-free water is used as a diluent if not otherwise specified.

Determinations of sulfates in chemicals and on glassware should be negative.

Reagents.

1. Benzidine chloride, 2.0071 gm. dissolved in 500 cc. of 0.2 N hydrochloric acid. 1 cc. of this solution is equivalent to 1.5 mg. of sulfate. From this solution, by dilution, working standards are prepared which contain 0.15, 0.03, and 0.015 mg. of sulfate equivalents. The diluent is always 0.2 N hydrochloric acid.

2. 20 per cent trichloroacetic acid.

3. Commercial hydrogen peroxide, a 1:5 dilution prepared when ready to use. Ordinary distilled water may be used as diluent.

4. A solution of 0.5 per cent ferric chloride in ordinary distilled water. Fresh solutions should be prepared weekly.

5. Pure acetone (redistilled if necessary).

6. Benzidine base, a 0.5 per cent solution in acetone. This solution should be discarded as soon as a yellow color develops.

Inorganic Sulfates of Serum.

Samples of blood collected in dry tubes which do not contain an anticoagulant are allowed to stand until clots form and then are centrifuged. 3 cc. of the serum are placed in a 15 cc. centrifuge tube with an equal amount of water (sulfate-free); to this, 3 cc. of 20 per cent trichloroacetic acid are added, and finally, enough water to make a total volume of 15 cc. The contents of the tube are thoroughly mixed and centrifuged for 5 minutes. 5 cc. of the supernatant fluid are added to, a second tube containing 10 cc. of the solution of benzidine base. The tube is capped and allowed to stand for 30 minutes or longer and then is centrifuged for 30 minutes at 3000 revolutions each minute. It is important to cap the tubes to prevent evaporation of the acetone before and during the centrifuging. The supernatant fluid is poured off and the tube is inverted for 3 minutes on a dry filter paper. The mouth of the tube is dried and 15 cc. of pure acetone are added; the precip-

itate is broken up with a fine pointed glass rod and is washed in the acetone. The tube is capped and centrifuged the second time for 15 minutes at 3000 revolutions each minute. The acetone is poured off and again the tube is inverted and allowed to drain on a filter paper, this time for 5 minutes. The lip of the tube is wiped with dry filter paper and 2 cc. of 0.2 N hydrochloric acid are added. If shaking does not dissolve the precipitate, the tube is warmed gently over a burner but the contents are not allowed to boil. A heated tube must be allowed to cool or must be cooled under running water before proceeding. 6 cc. of water are added, then 1 cc. of dilute hydrogen peroxide, and 1 cc. of the ferric chloride solution, and the contents of the tube are thoroughly mixed and allowed to stand for 5 minutes. After about 5 minutes the color is fully developed and remains constant until about 10 minutes have passed. If readings cannot be made during the second 5 minutes, 0.5 cc. of concentrated hydrochloric acid may be added to both the standards and the unknown. The hydrochloric acid prevents rapid fading of the colors.

Standards should be prepared simultaneously with the unknowns. It is important to have the acidity the same in the standard and in the unknown. For example, if 1 cc. of the standard containing 0.03 mg. of sulfate equivalent is used, 1 cc. of 0.2 N hydrochloric acid must be added because 2 cc. of 0.2 N hydrochloric acid were added to the unknown.

Calculation of Results.

$$\frac{S}{U} \times W \times 100 = \text{mg. of } SO_4 \text{ for each 100 cc. of serum.}$$

S = reading of the standard.
U = reading of the unknown.
W = SO_4 equivalent of the standard used.

Blood serum containing large amounts of sulfates must be diluted more than 1:5, depending on the amount of sulfates present.

It is important to select centrifuge tubes with a fine pointed conical bottom, for if tubes with a dome-shaped bottom are used, the precipitate is likely to be lost during the process of washing.

Inorganic Sulfates in Urine.

The procedure is exactly like that used in blood serum except for the dilution of the urine. 3 cc. of the urine are added to 3 cc. of water (sulfate-free) in a centrifuge tube of 15 cc. capacity, and to this are added 3 cc. of 20 per cent trichloroacetic acid, and finally enough water to make a total volume of 15 cc. This clears the urine of albumin if any is present. From this 1:5 dilution of the urine, further dilutions are made according to the amount of sulfates present; usually at least a 1:10 dilution is necessary. 5 cc. of the diluted urine are added to 10 cc. of the benzidine base solution and the procedure is exactly as that employed with blood.

If the urine contains a large amount of phosphates, it may be necessary to remove the phosphates; this may be done after the method of Fiske (5). When the phosphates are removed, the urine should be made slightly acid before precipitation of the sulfates.

Allowance for dilution is made in the calculation of the results.

$$\frac{S}{U} \times W \times \text{dilution} \times \text{volume of urine} = \text{mg. of } SO_4.$$

Total Sulfates in Blood Serum and Urine.

The procedure is the same as that for inorganic sulfates in serum and urine except that the filtrate is hydrolyzed with hydrochloric acid. 2 drops of concentrated hydrochloric acid are added to the 5 cc. of the filtrate contained in the centrifuge tube. The tube is placed in a beaker of boiling water for 15 minutes. At the end of 15 minutes, the tube is allowed to cool, or is cooled under running water, before the benzidine base solution is added. On addition of the benzidine base solution a dense turbidity forms. This turbidity is due to the acetone and hydrochloric acid and disappears as soon as the contents of the tube are thoroughly mixed. From this point on, the procedure is the same as that in determining inorganic sulfates.

The difference between the amount of the inorganic and total sulfates is the amount of conjugated sulfates present.

Procedures for Various Body Fluids.

3 cc. of the fluid produced in the course of the edematous condition from the peritoneum, thorax, or other regions are placed in

TABLE I.

Recovery of Sulfates from Solutions with Known Content of Ammonium Sulfate.

Theoretic amount of SO₄.	Amount of SO₄ recovered
mg. per cc.	*mg. per cc.*
0.149	0 143
0.149	0 143
0.149	0 140
0 059	0.056
0 059	0 055
0.059	0.058
0.029	0.026
0.029	0.029
0.029	0.026

TABLE II.

Recovery of Sulfates from Solutions with Known Content of Sulfuric Acid.

Theoretic amount of SO₄.	Amount of SO₄ recovered.
mg. per cc.	*mg per cc*
0.149	0.146
0.149	0.147
0.149	0.144
0.059	0.050
0 059	0.051
0.059	0.050
0.029	0.020
0.029	0.021
0 029	0 023

TABLE III.

Recovery of Added Inorganic Sulfates from Human Blood Serum.

Sulfates of blood serum.	Sulfates added.	Sulfates recovered.
mg. per cc.	*mg. per cc.*	*mg. per cc.*
0.007	0.010	0.016
0.007	0.020	0.026
0.007	0.030	0.037
0.007	0.007	0.010
0.007	0.005	0.011
0.007	0.004	0.010
0.007	0.004	0.010

a 15 cc. centrifuge tube, and to this are added an equal amount of water (SO₄-free) and 3 cc. of 20 per cent trichloroacetic acid,

TABLE IV.

Inorganic, Total, and Conjugated Sulfates in Urine.

Values are expressed in mg. of SO₄ in each cc. of urine.

Determina- tion No.	Gravimetric determinations.			Colorimetric determinations.		
	Inorganic.	Conjugated.	Total.	Inorganic.	Conjugated.	Total.
1	1.24	0.24	1.48	1.26	0.24	1.50
2	0.80	0.04	0.84	0.88	0.16	0.91
3	1.60	0.33	1.93	1.62	0.31	1.93
4	2.84	0.20	3.04	2.90	0.13	3.03
5	0.92	0.05	0.97	0.98	0.03	1.01
6	2.13	0.07	2.20	2.15	0.17	2.32
7	1.81	0.13	1.94	1.79	0.11	1.90
8	2.18	0.13	2.31	2.10	0.15	2.24
9	1.72	0.20	1.92	1.68	0.22	1 90
10	2.41	0.21	2.62	2.40	0.21	2.61

The last four determinations were made after the phosphates were removed, by the method of Fiske.

TABLE V.

Normal Values for Sulfates of Human Blood Serum.

Values are expressed in mg. of SO₄ in each 100 cc. of blood serum.

Inorganic sulfates.	Total sulfates.	Conjugated sulfates.
1.2	3.7	2.5
0.9	2.9	2.0
1.5	2.3	0.8
0.5	3.5	3.0
1.1	3.8	2.7
1.4	4.1	2.7
1.3	4.3	3.0
1.8	4.5	2.7
1.1	3.8	2.7
1.5	4.3	2.8

and the whole is mixed. Water (SO₄-free) is now added to make a total volume of 15 cc. These fluids often contain so little in bulk of protein that in order to obtain a clear solution after centri-

fuging, the tube should be allowed to stand for 30 minutes or longer.   The procedure is the same as in blood from this point.

Comment.

The recovery of sulfates from solutions with known contents of sulfate, from serum, and the comparison of the colorimetric method with the gravimetric method are shown in Tables I to V.   In Table IV it will be noted that the first seven determinations show that results with the colorimetric method are higher than those with the gravimetric method.   The phosphates were not removed from these samples because at this time it was thought that the dilutions were sufficient to make insignificant the amount of phosphates present.

In order to use the method for clinical studies, it was necessary to establish normal values for the sulfate content of the serum. Healthy persons were selected and the blood was drawn from 3 to 4 hours after the morning meal.   The results are shown in Table V.

In the past, investigators have not agreed as to whether there were conjugated sulfates in the blood.   Browinski (1) cited experiments which he believed proved the absence of such compounds in the blood of the horse.   Heubner and Myer-Bisch (6) believed that conjugated sulfates existed in the blood but did not offer objective evidence.   Kahn and Postmontier (10) considered that three forms of non-protein sulfur exist in the blood (inorganic, as sulfate conjugated, and neutral sulfur).   Denis, in her first work (4), was unable to demonstrate conjugated sulfates in the blood of human beings; she later demonstrated their presence by using trichloroacetic acid for precipitation of proteins instead of her original technique.   Her later results are confirmed by the present method in this study.

SUMMARY.

The colorimetric method, the principle of which was originally given by Hubbard, has been checked after making the changes which produced the most consistent results and has been found to be a microchemical method which is adaptable for clinical uses.

Additional procedures are described for the determination of total and conjugated sulfates in the blood serum and for total,

inorganic, and conjugated sulfates in the urine and in the fluids, which in edematous conditions collect in the peritoneal cavity, thorax, and elsewhere.

Normal values for the total, inorganic, and conjugated sulfate content of the blood serum as determined by this method are given.

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THE EFFECT OF THE POSITION OF SUBSTITUTION ON THE APPARENT DISSOCIATION CONSTANTS OF CERTAIN AMINO ACIDS.*

By CARL L. A. SCHMIDT, W. K. APPLEMAN, AND PAUL L. KIRK.

(From the Division of Biochemistry, University of California Medical School, Berkeley.)

In a recent paper, MacInnes (1) has shown that if the logarithms of the ionization constants of certain hydroxyl- and halogen-substituted acids be plotted against the reciprocal of distance, the relationship is expressed by a straight line. It interested us to apply this method of plotting to the apparent acid and basic dissociation constants of certain amino acids. The data presented show that the relationships between the apparent acid and basic dissociation constants and the reciprocal of distance are also straight line functions.

The technique used in preparing the titration curves and the method employed in calculating the apparent dissociation constants were the same as those which have been described in previous papers from this laboratory (2). All determinations of pH were carried out at 25°. The dl-α-amino-n-valeric acid was an Eastman Kodak Company product, the γ-amino-n-valeric acid was prepared in the chemical laboratory of the University of Illinois, and the δ-amino-n-valeric acid was obtained from Kahlbaum. In place of the β-amino-n-valeric acid, we used β-alanine which was a Schuchart product. All of the compounds were recrystallized and dried over phosphorus pentoxide before use. The substitution of β-alanine for the β-amino-n-valeric acid appears justifiable. Its dissociation constants are probably very nearly the same as those of the corresponding valeric acid. Simms' (3) values for α-alanine are: $K'_a = 1.9 \times 10^{-10}$, $K'_b = 2.26 \times$
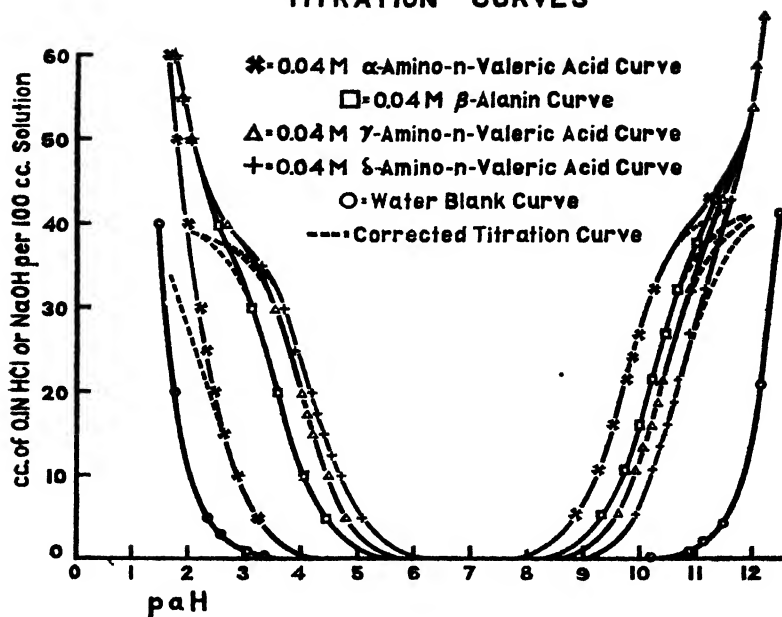
TITRATION CURVES
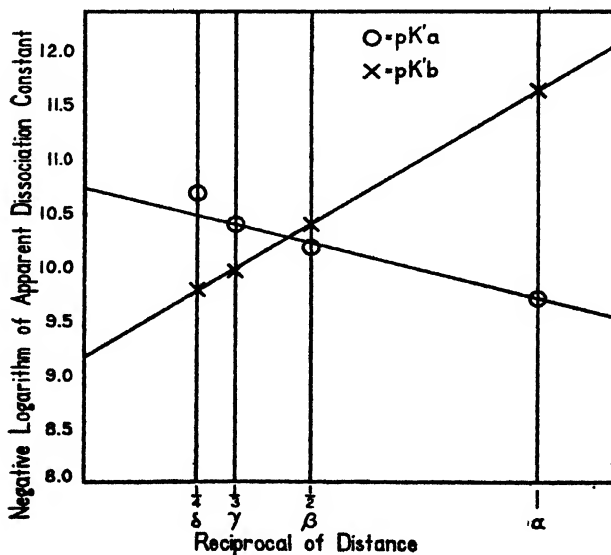
Fig. 1.

Fig. 2.

10^{-12}. They do not differ materially from the values for α-amino-valeric acid.

Fig. 1 shows the titration curves of β-alanine and the three amino-n-valeric acids together with the water blank curve. The graphs in Fig. 2 show that within certain limits of error the relationship between the logarithms of the apparent acid and basic dissociation constants and the reciprocal of distance can be expressed by straight lines. The slope of the curve in which values for K'_a are plotted is very much less than the corresponding curve for K'_b.

In Table I the apparent acid and basic dissociation constants for the four amino acids have been tabulated. It may be pointed out that our values for β-alanine differ slightly from those which

TABLE I.

Apparent Dissociation Constants.

	K'_a	K'_b	pI $= \frac{1}{2}$ (pK$'_a$ + pK$_w$ − pK$'_b$)[*]
α-Amino-n-valeric acid	1.9×10^{-10}	2.3×10^{-12}	6.04
β-Alanine	6.45×10^{-11}	3.98×10^{-11}	6.89
γ-Amino-n-valeric acid......	3.99×10^{-11}	1.06×10^{-10}	7.21
δ-Amino-n-valeric "	2.04×10^{-11}	1.62×10^{-10}	7.45

[*] The value for K_w has been assumed to be 1.005×10^{-14}.

have been reported by Ley (4). He found $K'_a = 7.1 \times 10^{-11}$ and $K'_b = 5.1 \times 10^{-11}$.

In the computations of MacInnes it was assumed that the aliphatic carbon chains are straight or at least that the distance, d, increases by regular increments as the substituent is moved out on the chain. Without offering any experimental evidence he states, "If, however, a basic group such as NH_2 is substituted in the chain, this group and the carboxyl group can apparently attract each other, producing distorted molecules. Thus a basic group far out on a carbon chain may, by bending, get very near the carboxyl group, resulting in a lowering of the ionization constant and possibly inner salt formation." While our results admittedly do not serve as a criterion to answer the question as to whether bending of the carbon chain occurs, they nevertheless give no

indication that this distortion, if present, affects the dissociation constants. In this connection, the experiments of Dunn and Schmidt (5) may be cited. They found that the time required for the amino group to react quantitatively with nitrous acid increases fairly regularly as the amino group moves away from the carboxyl in straight chain amino acids.

SUMMARY.

1. The apparent acid and basic dissociation constants of α-amino-n-valeric acid, β-alanine, γ-amino-n-valeric acid and δ-amino-n-valeric acid have been determined.

2. It has been shown that if the logarithms of the apparent dissociation constants of these amino acids are plotted against the reciprocal of distance of the amino groups the curves are straight lines.

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THE EXCRETION OF AMMONIA AND UREA BY THE GILLS OF FISH.

By HOMER W. SMITH.

(From the Department of Physiology, University and Bellevue Hospital Medical College, New York.)

A very small total nitrogen excretion by way of the urine appears to be typical of fish. This statement is attested by the analysis of *Lophius* urine reported by Denis (1), Marshall and Grafflin (2), and Grollman (3) and by the analysis of the urines of Mediterranean fish reported by Edwards and Condorelli (4). Further evidence is presented in Table I, in analyses of urines removed or collected by catheter from fresh and salt water fish, both fasted and in the active process of digestion. In Table I, Samples 1 to 5 comprise urines removed from the bladders of the goosefish, *Lophius piscatorius*, shortly after death. These fish had been caught in pound nets 2 to 4 hours previously, and on dissection the stomachs were found to be literally gorged with partially digested food. In one instance, between fifteen and twenty partially digested fish, weighing in all at least 2 kilos, were removed from the stomach of a 10 kilo fish. In all instances, there could be no question that active digestion and absorption were going on. Yet the total urinary nitrogen is but slightly greater than in the urines of *Lophius* after 3 days of fasting (Table I, Samples 6 and 7); these fish on dissection were found to be entirely free of food residues.

A similar low urinary nitrogen prevails in other species, on which typical data are given in Table I, the nitrogen being lower, in general, in the urines of fresh water fish than in the urines of marine fish.

There is, moreover, in all these data a peculiar distribution of nitrogen. The principal nitrogenous constituent identified here is creatine. This substance constitutes an exceptionally large

TABLE I.

Nitrogen Distribution in Fish Urine.

Urines 1 to 12 inclusive were removed from bladder shortly after death; others were collected by retention catheter. Nitrogen figures are given in mg. per 100 cc. of urine.

Sample No.		Total N.	Urea N.	Uric acid N.	Ammonia N.	Amino acid N.	Creatinine N.	Creatine N.	Rest N. per cent	Urine per kilo per hr. cc.	Remarks.
	Marine.										
	Lophius piscatorius.										
1		59.0	2.1	0.6	1.8	8.8	0.0	23.0	38.4		Well fed.
2	"	56.0	2.0	0.4	1.5	10.4	0.0	26.0	28.0		"
3	"	37.1	0.6	0.4	2.0	7.4	0.0	7.8	51.0		"
4	"	52.5	0.8	0.6	1.6	16.4	0.0	22.6	20.0		"
5	"	50.0	1.0	0.9	1.0	17.0	0.0	20.0	20.2		"
6	"	60.0	0.5	0.5	0.5	6.0	0.0	21.2	51.9		Fasted 3 days.
7	"	31.0	0.9	1.1	0.7	0.9	0.0	17.2	33.0		" 3 "
8	*Gadus callarias.*	89.4	10.1	1.5	0.0	13.2	2.0	45.6	19.0		"
9	"	140.0	9.1	2.4	0.0	30.0	3.0	77.0	15.2		"
10	*Sphaeroides maculatus.*	96.8	2.4	0.8	5.0		2.1	54.8	32.8		Feeding.
11	"	174.2	33.3	2.0	10.8	35.9	8.7	68.0	9.1		"
12	"	16.9	0.3	0.2	0.0		0.0	2.1	84.6		"
	Fresh water.										
13	*Anguilla crysypa.*	77.0	8.2	1.0	9.0		2.0	14.8	55.9	0.63	Fasted.
14	"	11.1	2.6	0.6	2.6		0.1	3.3	17.1	1.46	"
15	*Cyprinus carpio.*	49.1	Trace.	0.3	10.6		1.4	16.5	41.3	1.39	"
16	"	60.0	"	Trace.	2.0		0.0	10.9	78.5		"
17	"	27.2	1.0	"	Trace.		0.0	8.6	64.6	1.37	"

18	Cyprinus carpio.	12.5	Trace.	Trace.	0.0		0.0	8.5	32.0		Fasted.
19	Carassius auratas.	51.6	"	"	3.2		0.0	9.9	74.5	2.7	"
20	"	28.6	"	0.3	4.5		0.0	3.4	71.3	2.2	"
21	Ictalurus furcatus.	21.5	5.3	0.0	5.9	4.3	0.6	1.1	16.9		Feeding.
22	Archosargus probatocephalus.	18.7	4.3	1.2	1.8	2.8	1.1	1.8	25.1		"
23	"	16.6	3.8	1.4	1.4	3.3	1.5	1.0	26.4		"
24	Epinephalus morio.	37.4	11.5	0.0	0.0	2.4	0.9	5.5	33.7		"
25	Amiatus calva.	11.5	1.8	2.6	3.7	0.1	0.2	2.2	7.8	0.6	Fasted.
26	Lepidosteus osseus.	12.1	0.6	0.0	4.1	0.5	0.1	0.4	53.0	0.8	"

fraction of the total nitrogen, not because the creatine concentration is unusually high, but because the total nitrogen is unusually low.   Grollman (3) has recently shown that in the case of *Lophius*, the unidentified nitrogen (which is a large fraction in all fish urines examined by us) is trimethylamine oxide.

We were led by the above and other facts to suspect that nitrogen, possibly as ammonia or urea, was being lost from the body by some route other than the kidneys, and the gills appeared to offer the most probable avenue for this escape.

Methods.

In planning experiments designed to detect such extrarenal loss of nitrogen, it seemed desirable to separate any possible excretion by the gills from that of the skin or gastrointestinal tract.

To this end the fish was placed in a box divided into two chambers by a piece of rubber dam, thus separating the water circulating through the gills from that around the rest of the body.   A rectangular wooden trough was built, slightly larger than the fish, and the seams and sides caulked with paraffin to make it watertight.   This trough was then sawed in two parts, a short part for the head and a long part for the body. The adjacent edges of the two parts of the trough were coated with a vaseline-paraffin mixture.   A hole about 1 inch in diameter was cut in a piece of strong rubber dam 6 inches square, and the rubber was slipped over the head of the fish and placed just behind the pectoral fins.   The fish was placed in position with the apron spread between the ends of the opposite parts of the trough, and these parts were then brought together and firmly fastened with furniture clamps. Or, as is somewhat easier, the rubber dam was put into place and the ends of the trough fastened together, after which the fish was slipped head first through the hole in the dam and into position. In later experiments two superimposed layers of rubber dam were used to reduce leakage around the body of the fish.   Sufficient water was put in the two chambers thus made to cover the fish, and both chambers were aerated with a stream of ammonia-free air; the front chamber supplied the fish with oxygen, and the rear chamber served as a control.

The urine could be collected separately by a glass retention catheter sewed in the urinary papilla.   The open end of the cathe-

ter carried a light rubber bag to contain the urine. The shape and size of the catheters varied necessarily from fish to fish, but in general they were L-shaped; the shorter arm was inserted in the papilla and fastened with a purse-string ligature, and the longer arm was attached to the anal fin so that the greater part of the weight was removed from the papillary insertion.

There is little difficulty in keeping fresh water fish in this divided box for 24 hours or longer, if the water is well aerated and the gill movements are not impeded. It is difficult to prevent leakage between the chambers, the occasional movements of the fish and the constant respiratory currents back from the gills increasing the tendency for leakage of fluid to occur between the body of the fish and the rubber dam. This can be reduced to a minimum by the use of two layers of rubber dam, neither of which need constrict the fish sufficiently to cause apparent discomfort.

The methods of urinalysis were those commonly used, with slight modifications appropriate to the small concentrations of the urinary constituents Total nitrogen was determined by Koch and McMeekin's method (5) and direct Nesslerization. Ammonia was determined by extraction with permutit, as recommended by Folin and Bell (6). Urea was determined by decomposition with urease after extraction of the ammonia with permutit, the resulting ammonia being determined as above. (In the analysis of solutions in which the fish had been immersed, the same methods were used with larger samples (5 to 50 cc.).) Creatine was converted to creatinine by heating for 2 to 3 hours in a water bath with equal volumes of N HCl, after which the mixture was neutralized with N NaOH and the total creatinine, as well as the preformed creatinine, determined by Folin's method (7). A blank was included in all creatinine and creatine determinations. Uric acid was determined by Benedict's method (8), and amino acids by Folin's method (9). The inorganic constituents in Table III were determined in the concentrated solutions by commonly used micro methods (10).

The ammonia determinations on blood given in Table VI were made by Nash and Benedict's method (11). The fish were bled by cutting off the tail close to the anal fin, and 6 to 8 cc. of blood were collected over 25 mg. of ammonia-free potassium oxalate. 3 cc. of this oxalated blood were added, drop by drop, with slight agita-

tion, to 10 cc. of saturated ammonia-free Na_2CO_3 solution contained in a Nash-Benedict aeration tube, a drop of caprylic alcohol was added to prevent foaming, and the mixture aerated briskly at once for 10 minutes. Three wash bottles containing 5 per cent H_2SO_4 and one wash bottle containing 0.1 N HCl were used to remove ammonia from the air, which was pulled through by a suction pump. The ammonia was collected in 5 cc. of ammonia-free water to which had been added 6 drops of N HCl, and the solution was Nesslerized by the addition of 6 drops of Nessler's solution and compared transversely or in a colorimeter with $(NH_4)_2SO_4$ standards.

The important departure from Nash and Benedict's method was in the use of 10 cc. of saturated Na_2CO_3 solution for liberating the ammonia instead of 1 cc. of K_2CO_3-$K_2C_2H_2O_4$ solution recommended by them. This change in technique we found necessary because fish blood jells on the addition of alkali. By adding the blood drop by drop to a larger quantity of alkali the protein precipitation takes a fine particulate form which does not interfere with rapid aeration.

We never succeeded in reducing the blank determinations below 0.001 mg (corresponding to 0.02 mg. per 100 cc. on a 5 cc. sample) in spite of repeated trials and precautions over a period of several weeks. Consequently a blank determination was made just before and just after a series of blood determinations and appropriate corrections were made. Since the normal ammonia concentration in fish blood appears to be somewhat greater than that of mammalian blood, a blank of this magnitude, when carefully controlled, is not a serious impairment.

In all analyses a second aeration of 10 minutes was made to determine if all the ammonia had been removed. In only two out of a dozen instances was the amount collected during the second aeration greater than the blank. From this fact we do not believe that ammonia formation occurs in fish blood after addition to the Na_2CO_3 solution. On the other hand, the ammonia content of oxalated blood may increase four- or fivefold in an hour after being drawn when no alkali is added, especially, we think, if hemolysis occurs.

| The fish used were the carp, *Cyprinus carpio*, and large specimens of the common goldfish, *Carassius auratus*. The carp were

procured in the local markets and had not, in all probability, eaten for several weeks. For the goldfish we are indebted to the New York Aquarium. These had been eating regularly, and when kept in warm water would eat oysters in the laboratory tanks.

Results.

Within 10 minutes after putting water on the fish in this "divided box," the presence of ammonia in the front chamber, *i.e.* around the gills, can be demonstrated by Nesslerizing 5 cc. of the solution. The ammonia concentration steadily increases around the gills, while no trace of ammonia may appear in the back chamber around the body of the fish for several hours. (See Table II, Samples 1 to 4.)

In several experiments running 18 to 24 hours, ammonia never appeared in appreciable quantities in the rear chamber. This fact excludes the possibility that in these particular experiments this substance was excreted by the skin or gastrointestinal tract in significant quantities or that it was formed by oxidation or by ammoniacal fermentation of substances dissolving from the skin. The fact that in other experiments some ammonia does appear in the rear chamber (though always less in amount than in front) is attributed to leakage of fluid between the dam and the body of the fish. Further evidence that the appearance of ammonia in the front chamber was not due to ammoniacal fermentation was obtained by allowing the water removed from around the fish, at the conclusion of an 18 to 24 hour experiment, to stand at room temperature. In some instances a slight increase, in others no increase, of ammonia occurred during the 2nd and 3rd day; and where the ammonia did increase in amount, the quantity formed was considerably less than that which appeared during the first day when the fish was in the solution. If the ammonia were the result of bacterial action, it would be expected that more ammonia would be liberated during the 2nd day, when the bacterial infection was well developed, than during the 1st day when the infection was light.

With the elimination of secretion from the skin and gastrointestinal tract and the formation of ammonia by oxidation or bacterial decomposition, the only explanation that remains for the rapid ap-

TABLE II.

Excretion of Nitrogen by Gills and Kidneys of Fish.

Sample No.		Weight.	Time.	Front chamber.			Back chamber.			Urine.				Urine per kilo per hr.	N per kilo per 24 hrs.	Total N in urine.
				Vol-ume.	NH₃ N	Urea N.	Vol-ume.	NH₃ N	Urea N.	Vol-ume.	Total N.	NH₃ N	Urea N.			
		gm.	hrs.	cc.	mg.	mg.	cc.	mg.	mg.	cc.	mg.	mg.	mg.	cc.	mg.	per cent
1	Carassius auratus.	235	24.0	280	21.7	4.60	420	0.0	0.0	14.5	4.7	1.61	0.3	2.4	132	15.1
2	"	255	19.0	250	7.8	0.86	425	0.0	0.0	7.0	1.23	0.47	0.07	1.4	49	12.5
3	Cyprinus carpio.	420	16.3	215	9.3	2.20	260	0.8	0.4	2.0	0.3			0.3	46	2.7
4	"	600	18.0	250	19.6	2.50	560	0.6	Trace.	4.0	1.6	0.14	0.0	0.2	54	6.9
5	" "	1500	17.0	1100	29.2	2.80	1800	1.5	Trace.	In back chamber.					29	4.5
6	" "	1400	18.0	1290	52.2	5.00	1540	9.1	"	"					59	13.7
7	Carassius auratus.	220	18.0	615	15.2	2.1	580	2.2	0.1	"					119	11.6
8	" "	330	22.5	180	4.8	0.46	300	Tr.	?	"					17	?
9	Cyprinus carpio.	530	15.3	930	38.0	4.06	In one chamber.			40.0	6.11	3.7	0.99	4.9	142	12.7
10	"	315	6.0	530	7.5	0.78	"	"	"	4.0	0.74	0.37	Trace.	2.11	38	24.5
11	"	530	21.6	960	45.0	3.72	"	"	"	36.0	6.45	1.06	0.66	3.15	115	11.7
12	"	500	18.5	1800	46.8	2.40	"	"	"	18.5	1.25	0.46	Trace.	2.00	131	2.5

pearance of ammonia in the front chamber is that this substance is excreted by the gills in considerable quantities.

The excretory rôle of the gills is substantiated by the finding of urea in the water removed from the front chamber. The ratio of urea to ammonia in the branchial excretion is low in comparison with that obtained in the urine of mammals, a fact suggesting that some of the urea may have been decomposed by bacterial action, during the experiments. That such destruction was relatively small is indicated by experiments in which added urea, when aerated for 24 hours in solutions previously used in experiments with the divided box, shows only 1.5 to 17 per cent decomposition in original concentrations ranging from 20 to 5 mg. of urea N per 100 cc. From this it is inferred that the relative proportions of ammonia and urea in these solutions represent approximately the proportions in which these substances are excreted by the gills.

Comparison of the branchial with the urinary excretion shows that 6 to 10 times as much ammonia is excreted in the same interval by the gills as in all nitrogenous substances together by the kidneys. Since ammonia and urea may be almost entirely absent from the urine, the relative amounts of these substances excreted by the gills and kidneys show a still greater difference.

One criticism which may be made of the above experiments is that the rate of urine excretion may be reduced considerably below normal. These same fish, when swimming free in the tank with the retention catheter sewed in place, excrete from 1.0 to 5.0 cc. of urine per kilo per hour, whereas in the divided box this excretion may fall to a much lower figure. We believe that this is principally due to the closing of the catheter by the tissues of the urinary duct, with the consequent obstruction of urine flow, an event more likely to occur when the fish is practically immobilized than when swimming free. It is also possible that the constriction of the rubber dam by retarding circulation reduces the rate of urine formation. No doubt an anuria from any cause would lead to a rise in the ammonia and urea concentration of the blood; but it does not seem probable that this elevation would lead to the extensive excretion of these substances by the gills if such excretion did not in some measure occur under normal conditions. It may be noted, moreover, that the observed branchial excretion

would exceed by several fold the maximal ammonia excretion in the urine in the same period of time, even when this maximal excretion is figured from a maximal flow of urine (about 5.0 cc. per kilo per hour) and a maximal ammonia content (about 10.0 mg. of NH_3 N per 100 cc.).

But in view of this objection, it seemed that experiments with the divided box without the retention catheter, or experiments with the retention catheter but in which the fish was allowed to swim free, might be more desirable since the chances of anuria

TABLE III.

Cyprinus carpio, 1.5 Kilos, in Divided Box 24 Hours, with Urine Collected by Retention Catheter.

	Front chamber (volume = 850 cc.).	Back chamber (volume = 1470 cc.).
	mg. per 100 cc.	*mg. per 100 cc.*
NH_3 N	2.35	0.3
Urea N	0.318	0.0
Uric acid N	Trace.(?)	0.0
Creatinine N	0.016	0.022
Creatine N	0.053	0.030
	mм per l.	*mм per l.*
B^+	1.34	1.32
K	0.33	0.19
Ca	0.18	0.22
Mg	0.10	0.03
SO_4	0.17	0.16
Cl	0.44	0.62
CO_2	1.71	0.20

would be be reduced. The latter type of experiment appeared especially favorable, inasmuch as urine formation is abundant, and previous experiments had ruled out the possible excretion of any considerable quantities of nitrogen by the skin or gastrointestinal tract.

The results of both types of experiments are reported in Table II, Samples 5 to 12. The results show that even when the urine formation is abundant, the branchial excretion exceeds the urinary excretion by several fold.

In several instances solutions obtained in experiments with the

divided box were concentrated by evaporation on a hot plate, and
the concentrated solutions examined for uric acid, creatinine,
creatine, potassium, and chloride.   The results of such experi-
ments, one of which is given in Table III, do not indicate that any
of these substances escape from the body by way of the gills in sig-
nificant quantities.   Creatine and potassium may be present in
larger quantities in the front chamber, but their appearance can
be explained by hemorrhage following trauma to the gill capil-
laries.   Merely removing a fish from water will sometimes cause
slight branchial hemorrhage.

Since some of the fish used in the above experiments had not
eaten for several days (Samples 2–6, 8, and 10) the ammonia and
urea excretion, which averages 42 mg. of N per kilo per 24 hours,
approaches a basal level, in so far as such a state can be realized

TABLE IV.

Urea and Ammonia Excretion of Frog, Rana catesbiana.

Weight.	Tempera- ture.	Time.	Volume.	NH₃ N	Urea N.	N per kilo per 24 hrs.
gm.	*°C.*	*hrs.*	*cc.*	*mg.*	*mg.*	*mg.*
427	18	45.5	360	9.7	18.3	34.5
427	18	46.5	670	15.4	12.0	33.0
498	18	45.5	280	23.2	9.2	34.3
498	18	46.5	600	45.0	6.6	53.5

with these animals which are continuously active.   It was thought
worth while, therefore, to determine the nitrogen excretion of
frogs for comparison, especially since our figures appear to be
rather high.   Pryzlecki, Opienska, and Giedroyc (12) have shown
that in the frog a large fraction of the non-protein nitrogen may be
lost by way of the skin; but since figures suitable for comparison
with ours are not available, we have determined the ammonia
and urea excretion of the bullfrog, *Rana catesbiana*, in a few in-
stances.   The frogs had been fasted for at least 3 months.   They
were placed individually in glass jars just large enough to contain
them comfortably and with just sufficient water to cover them.
The bladders were emptied before and at the conclusion of the
experiments and the urine added to the water.   Ammonia and
urea were determined directly on the mixed urine and water by

the use of permutit.   The results of these experiments are given in Table IV.   The total ammonia and urea excretion, about 35 mg. of nitrogen per kilo per 24 hours, compares favorably with the lowest figures obtained on fish.

That we might evaluate the relative importance of the branchial excretion as compared with the urinary excretion, the distribution of nitrogen in the branchial and urinary excretions has

TABLE V.

Branchial and Urinary Nitrogen Excretion in the Fresh Water Carp,
Cyprinus carpio.

Fish 1. Weight 500 gm., in 1260 cc. of water 23 hours at 18.5°.   Excreted 58 cc. of urine or 5.0 cc. per kilo per hour.

Fish 2. Weight 368 gm., in 1360 cc. of water 21.5 hours at 18.5°.   Excreted 45 cc. of urine or 5.7 cc. per kilo per hour.

	Fish 1.			Fish 2.		
	mg. per 100 cc.	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*
Water.						
Urea N.....................	0.34	4.3	5.0	0.31	4.2	6.0
NH₃ N.........................	3.70	46.6	54.1	3.18	43.2	62.0
Amine N*.....................	0.16	2.0	2.7	0.40	5.4	7.8
Undetermined N..............	1.91	24.1	28.0	0.78	10.6	15.2
Urine.						
Urea N.......................	1.1	0.64	0.7	1.0	0.45	0.7
NH₃ N........................	2.75	1.60	1.9	2.24	1.01	1.5
Uric acid N...................	0.28	0.16	0.2	0.33	0.15	0.2
Creatinine N..................	0.0	0.0	0.0	0.0	0.0	0.0
Creatine N................. ..	9.7	5.63	6.5	6.8	3.06	4.4
Amine N*.....................	1.36	0.79	0.9	2.64	1.18	1.7
Undetermined N...............	0.21	0.12	0.2	1.1	0.50	0.7
Total N excreted................		86.1			69.6	

* Base volatilized on boiling with zinc dust and NaOH minus ammonia.

been determined more completely in two instances (Table V). The excretion of amine nitrogen has also been determined in these experiments.   The water was acidified with HCl and concentrated to 0.1 volume.   An aliquot was digested for 5 hours and finally distilled with an excess of NaOH and zinc dust, the volatile base being collected in 0.02 N HCl, and measured by titration with 0.02 N NaOH.   Assuming the volatile base to be monova-

lent, each cc. of 0.02 N HCl = 0.28 mg. of N. The difference betwcen this volatile base liberated by alkali and zinc and the ammonia in the distillate, as determined by Nesslerization, is designated as amine N. The amine nitrogen of the urine was determined in the same way on 10 cc. samples without preliminary concentration. Presumably a large fraction of the volatile nitrogen, in excess of ammonia, obtained in this manner is trimethylamine liberated from trimethylamine oxide, which Grollman (3) reports in the urine of *Lophius*. We have not attempted to identify this volatile base.

The total nitrogen is taken as the sum of the total nitrogen in the water and the total nitrogen in the urine. Figured in this way, it is seen that ammonia and urea account for the greater fraction of the total nitrogen. The urinary creatine, though constituting 50 per cent of the urinary nitrogen is only 8 per cent of the total nitrogen, indicating that this substance does not play an unusual rôle in the protein metabolism of fishes. Comparable quantities of creatine are excreted by birds (13, 14) and reptiles (15, 16). Large quantities of amine N are excreted by the gills as well as by the kidneys. This substance constitutes 3 to 10 per cent of the total nitrogen excreted.

The presence of amine or amine oxide nitrogen, the predominance of ammonia over urea, and the predominance of creatine over creatinine are the significant differences between the nitrogenous excretion of these fishes and of mammals.

The concentration of urea in the water around the gills has never in any of our experiments reached a value sufficiently great to suggest that the excretion of this substance by the gills involves anything more than diffusion from the blood through the gill membranes. The ammonia concentration, on the other hand, reaches such high values in most experiments that one is led to suspect secretory activity on the part of the gills for this substance. There also arises the question as to whether this ammonia is taken from the blood as such by the gills, or whether it is made from some ammonia precursor, as the work of Nash and Benedict (11) and of Bliss (17, 18) has indicated is the case in the mammalian kidney.

With the intent of testing these questions, a few sacrifice experiments have been performed in which the ammonia and urea

concentrations of the blood and water around the gills have been determined simultaneously.   It was clear at the outset that in the case of ammonia, if diffusion were the essential factor, the free NH_3 rather than NH_4OH or NH_4^+ would probably be the important component, and that a critical experiment would require an aqueous solution, bathing the gills, of approximately the same hydrogen ion concentration as the blood.   (If the solution were 0.3 pH more acid than the blood, twice as much ammonium salt would be required in the water to establish diffusion equilibrium

TABLE VI.

Simultaneous Concentrations of Urea and Ammonia in Blood and in Water around Gills at Conclusion of 18 Hour Experiments (Cyprinus carpio).

Nitrogen figures are given in mg. per 100 cc.

pH	Water.		Blood.	
	Urea N.	Ammonia N.	Urea N.	Ammonia N.
	0.0	0.0	1.92	
	0.0	0.0	1.40	
	0.0	0.0	2.10	
	0.30	2.60	3.12	
	0.58	3.11	1.86	
	0.41	2.98	5.55	
	0.0	0.0		0.11
	0.0	0.0		0.07
	0.0	0.0		0.31
	0.0	0.0		0.22
7.18	0.34	3.68		1.26
7.10	0.33	2.90		0.70
7.30	0.34	3.20		1.40
7.16	0.31	3.18		1.51

with respect to the free NH_3.)   Consequently in these experiments a small quantity of phosphate buffer was added to the water surrounding a catheterized fish, and the fish left with constant aeration of the water for 24 hours.   At the conclusion of the experiment the fish was immediately bled by cutting off the tail close to the anal fin, 5 to 8 cc. of blood collected over 25 mg. of potassium oxalate, and the ammonia concentration of the whole blood determined at once with every precaution recommended by Nash and Benedict (11).   The ammonia and urea content of the water

were determined as previously, and the hydrogen ion concentration by the quinhydrone electrode. The urea content of the blood was determined on a trichloroacetic acid filtrate after neutralization and extraction of ammonia with permutit. The urea was decomposed by urease and the ammonia determined by direct Nesslerization.

The results of these experiments are given in Table VI. The blood urea is so much higher than the water urea that one does not hesitate to ascribe its escape from the body to diffusion.

The blood ammonia, however, is always somewhat lower than the water ammonia. The difference is, in these experiments, accentuated by increasing the acidity of the water. We did not determine the pH of the blood in those fish analyzed for ammonia because of the limited quantity obtained, but on other fish the pH of the serum has ranged between 7.3 and 7.5. It should be noted that the higher salt content of the blood would tend to reduce the activity of the ammonium ion in the blood as compared with the water, and possibly alter the hydration of the free ammonia as well. These factors complicate the interpretation of these experiments, but we believe the difference in total ammonia in the blood and water is too small to justify the belief that secretory activity on the part of the gill membranes is involved in the escape of ammonia from the body by this route. In any case, the fact that the blood ammonia shows such a marked rise under these conditions is substantial evidence that the excreted ammonia is derived from blood ammonia and not from some ammonia precursor.

SUMMARY.

The urinary nitrogen of the fresh water carp and goldfish constitutes only a small fraction of the total nitrogenous substances excreted by these fish. Approximately 6 to 10 times as much nitrogen is excreted by the gills as by the kidneys. The branchial excretion consists largely if not entirely of the readily diffusible substances, ammonia, urea, and amine or amine oxide derivatives. The less diffusible substances (creatine, creatinine, and uric acid) are excreted by the kidneys.

The excretion of urea by the gills can unquestionably be explained by diffusion, and it appears probable that the excretion of

ammonia can be accounted for similarly without invoking secretory activity on the part of the gill membranes.

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OXIDATION-REDUCTION SYSTEMS OF BIOLOGICAL SIGNIFICANCE.

III. THE MECHANISM OF THE CYSTEINE POTENTIAL AT THE MERCURY ELECTRODE.

BY E. S. GUZMAN BARRON, LOUIS B. FLEXNÉR, AND L. MICHAELIS.

(From the Laboratory of Research Medicine, Medical Clinic, the Johns Hopkins University, Baltimore.)

INTRODUCTION.

It was pointed out in the first communication of this series (1) that cysteine in the absence of oxygen establishes a definitive potential at electrodes of mercury, platinum, and gold-plated platinum which, in agreement with the finding of Dixon and Quastel, is independent of the concentration of cystine. Further it was observed that the potentials at these three electrodes are well reproducible and are equivalent within the limits of experimental error. No satisfactory mechanism could be proposed for the potential and the attempted explanations of Dixon and Quastel (2), of Dixon (3), and of Kendall and Nord (4) were judged to be unsatisfactory.

It is well known that many substances other than cysteine give a very negative potential at mercury compared with the normal mercury potential. The negativeness of the potential in these instances is ascribed to formation of complex anions which contain mercury but which are practically undissociated. Several of these substances were studied with the idea that their behavior might be related to that of cysteine, and of these substances cyanide proved most fully analogous experimentally and theoretically. The first part of this communication, therefore, is given to observations on the behavior of cyanide and some other complex-forming anions in the presence of mercury. Some of these observations have long been known, some are new, but all are understandable in the light of tested and approved hypotheses.

The second part of the communication is concerned with a description of the behavior of cysteine under those experimental conditions imposed upon cyanide.

Mercury and Cyanide.

A cyanide solution is known to establish a negative potential at an electrode of metallic mercury. Immerwahr (5) measured, in presence of air, the potential of a 0.05 M cyanide solution in 1 M KNO_3 saturated supposedly with mercurous cyanide by addition of small amounts of mercurous nitrate, and found it equal to +0.269 volt referred to the normal hydrogen electrode. The potentials of solutions free of added mercury salts were unsteady and drifting and reached a final value only after a rather long time. Immerwahr's assumption that his cyanide solutions became saturated with mercurous cyanide on addition of small amounts of mercurous nitrate is not quite justified, as Sherrill pointed out (6). Complex mercurous compounds are practically unknown, and whenever their formation might be anticipated the mercuric complex and metallic mercury are found instead. In agreement with this a mercurous compound of cyanide is unknown and in its place mercuric cyanide arises.

Mercuric cyanide is practically completely undissociated; this is shown by its extremely low electric conductivity. There are consequently practically no Hg^+ or Hg^{++} ions in a solution such as is got when cyanide is in the presence of metallic mercury and the potential of the mercury against the cyanide solution cannot be referred to the concentration of mercury ions in solution (Haber (7), Clark (8)). When the mercury ion concentration in a cyanide solution is computed from the potential according to Nernst's equation, it is found to be so extremely low as to be almost without physical significance. The reaction responsible for the potential is best thought of in this way:

$$Hg + 2CN^- \rightleftharpoons Hg(CN)_2 + 2\epsilon \tag{1}$$
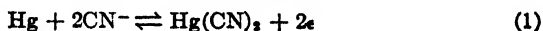
This is the chemical reaction which takes place reversibly at a Hg electrode in contact with a cyanide solution. The equation shows that mercury goes into the solution when mercury and a cyanide solution form the negative pole of a galvanic cell. This can easily be demonstrated. An electrode vessel containing a

mercury electrode and an M/15 solution of Na_2HPO_4 was thoroughly bubbled with purified nitrogen; then KCN was added. This half-cell was connected with a calomel electrode by an agar bridge saturated with KCl and the circuit completed through a milliammeter which showed a steady current of 0.4 milliampere. A control experiment was made by preparing a vessel in exactly the manner just described except that there were no electrical connections. The experiments were discontinued after 5 hours. The solution of the half-cell through which there was passage of current gave a heavy precipitate of HgS with H_2S, proving the existence of a mercury-cyanide complex. No mercury was demonstrable in the solution of the control experiment with open circuit.

The magnitude of the cyanide potential at mercury according to equation (1) above is a function of the concentrations of cyanide ions and of $Hg(CN)_2$ in solution. The reaction between metallic or ionic mercury and cyanide is unquestionably much more complex than that indicated by the equation. Sherrill speaks of the molecules $Hg(CN)_2$, $Hg(CN)_4$ and of the ions $Hg(CN)_2(CN)_2^-$ and $Hg(CN)_2CN^-$. All of these complexes may be present at the same time in equilibrium with each other but in ratios varying with experimental conditions. It is impossible to relate experimental measurements of the cyanide potential at mercury with or without addition of mercury ions to a mathematical formula without considering the equilibrium between the various complexes, but very little is known of this. Because of this difficulty no quantitative study was made of the cyanide potential.

The negative potential of the mercury-cyanide half-cell means that such a system has the properties of a reductant. This has been demonstrated in the following three ways.

1. Two flasks were arranged with purified nitrogen flowing through each. Into one flask was placed M/15 Na_2HPO_4, metallic mercury, KCN, and a few crystals of phenol indophenol. The dye was quickly reduced. The contents of the second flask were the same as those just given except that there was no mercury present; here there was no observable reduction of the dye.

2. It may be anticipated from the results of the dye experiment that KCN will consume oxygen in the presence of metallic mercury. Fig. 1 gives briefly the results of such experiments carried out in Warburg's micro respiration apparatus.

Cyanide, of course, is but one of several ions which give oxygen consumption in presence of mercury. Oxygen consumption is to be expected too when mercury is shaken with other solutions against which it has a sufficiently negative potential. The negativity of mercury against potassium salts of various anions
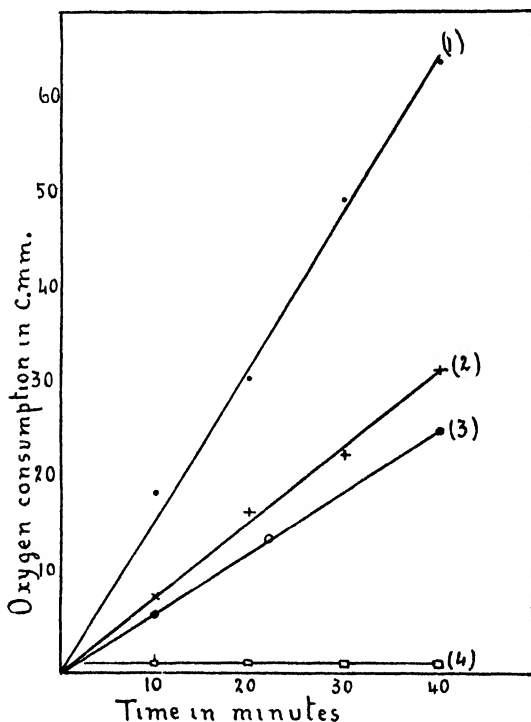
Fig. 1. Oxygen consumption, in presence of metallic mercury, of 1 M solutions of: (1) potassium cyanide, (2) sodium thiosulfate, (3) potassium iodide, (4) potassium chloride. pH of all solutions, 7.0. 2 cc. of solution + 0.2 cc. of metallic mercury in all experiments.

increases in the order: Cl, Br, SCN, I, CN, thiosulfate, H_2S, and consumption of oxygen is to be anticipated when mercury is in contact with any of these ions. Affinity is not the sole factor determining reaction velocity but, when other conditions are equal, the rate of consumption of oxygen by systems exposed

to air will be approximately a function of the negativity of the potential in the absence of oxygen. The anions mentioned above have been arranged in order of increasing negativity of potential at mercury and it is to be expected that this order is approximately the same for rate of oxygen consumption. This, indeed, was found to be true. For example, mercury shaken with KCl even at high concentration (4 M) shows no measurable oxygen consumption. With KI, oxygen consumption is negligible at 0.1 M concentration but becomes appreciable in 1 M solutions. With KCN, oxygen consumption is marked even at 0.01 M concentration.

3. A galvanic chain was made with an electrode of metallic mercury in contact with a solution of cyanide as one half-cell and an electrode of bright platinum in contact with a solution of methylene blue as the other half-cell. Both dye and cyanide were dissolved in M/15 Na_2HPO_4. After expulsion of air from the dye system, the two half-cells were joined through a milliammeter and an electric current seen to flow. The dye was completely reduced within an hour.

To summarize briefly, it will be recalled that cyanide causes a negative potential at mercury, that this potential is consequent upon the formation of a practically undissociated complex between cyanide and mercury, and that a solution of cyanide in contact with metallic mercury forms a reducing system which is able both to consume oxygen and to reduce a dye.

Mercury and Cysteine.

Hydrogen sulfide, like cyanide, forms an extraordinarily stable, practically undissociated compound with mercury. This compound, as in the case of cyanide, contains mercury in the mercuric state. To be sure a mercurous sulfide has been described but it is to be got only at very low temperatures and even then is a labile, transient product. Except for this rather unimportant exception, then, it can be said that mercuric sulfide is always formed whenever metallic mercury or a mercury compound of any kind is brought into contact with hydrogen sulfide.

It is to be expected that cysteine, a substitution product of hydrogen sulfide, reacts, in principle, in the same way with mercury as does hydrogen sulfide. Brenzinger (9) has described a white, crystalline compound formed by cysteine and mercuric

chloride. This complex is to be got too by shaking cysteine with
solid, red mercuric oxide; it appears in the form of beautiful, white
needles. Even when calomel is shaken with cysteine, the same
mercuric complex is obtained and with it there is obviously
metallic mercury, for the calomel solution becomes gray. The
reaction with mercurous nitrate is a little more involved. When
a very dilute solution of this salt is added to a cysteine solution, a
brown, cloudy precipitate forms which gradually fades away.
Addition of sufficient mercurous nitrate finally results in the
appearance of the white needles of mercuric cysteinate spoken of
above. The solution is again made gray by colloidal, metallic
mercury. The transient brown substance is probably a mercurous
compound of cysteine which is unstable and breaks down to yield
mercuric cysteinate and mercury.

These reactions demonstrate a great affinity between the SH—
group and the ions of mercury and it is not surprising that metallic
mercury behaves in the same way. Cysteine solutions have been
shaken in air with metallic mercury in the pH range from approxi-
mately 2 to 12. Cysteine dissolved in 0.01 M HCl, when shaken
in contact with mercury and in the presence of air, gives rise to
white, needle-shaped crystals, identical apparently with those
formed by cysteine and mercury ions. The crystals, when dis-
solved, give a strongly positive test for mercury with H_2S. The
same compound, judged by its crystalline structure and its content
of mercury, is got from cysteine solutions when shaken with
metallic mercury in standard acetate (pH 4.6) and Sörensen's
phosphate (pH 7.7). There is a striking difference in the result
at the pH of a borate buffer (pH 9.3) and of 0.1 M NaOH. Some
of the white, needle-shaped crystals are still to be found but
mixed with them are white, globoid crystals found more abun-
dantly at the higher pH of the 0.1 M NaOH than at the pH of the
borate buffer. These globoid crystals are of a compound of
mercury and cysteine and are easily shown to contain mercury.
In analogy with other mercury complexes, cysteine and mercury
give different complexes under different conditions. Brenzinger
has shown that several mercury complexes of cysteine exist and
that the composition of that complex studied by him most accu-
rately does not agree entirely with any of those simple formulæ

which may be anticipated.. The needle-shaped crystalline complex is obviously different from the globules. It is interesting to note that the latter are converted into the needles, when shaken at a pH 7 with metallic mercury.

It is certain, therefore, that metallic mercury enters into molecular combination with cysteine, that the composition of the compound consequent upon this combination is dependent on various conditions, especially on the pH, and that the reaction is progressive in presence of oxygen. It may be well to mention here that as a control the solubility of metallic mercury shaken in air was tested in standard acetate, phosphate, and borate buffers without cysteine. On no occasion was mercury to be demonstrated in the solution by the H_2S test under the same conditions which led to its appearance after addition of cysteine to the buffers.

There is no necessity to give in detail the data which prove cysteine solutions in the presence of metallic mercury to be reducing systems. This has been the thesis of Paper II of this series (10). It will be recalled that cysteine solutions in contact with mercury absorb oxygen over the wide pH range from 1 to 12 and that dyes also are reduced.

We come now to an interpretation of these last experiments. It is to be emphasized that mercury does not act as a catalyst as do ferrous salts, which accelerate the oxidation of cysteine to cystine, but that the reducing power of cysteine solutions in contact with mercury is consequent upon a stable combination between metal and cysteine. These observations are to be explained by this reaction:

$$2RSH + Hg \rightleftharpoons (RS)_2Hg + 2H \qquad (2)$$
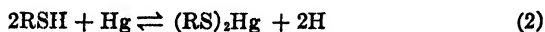
The reaction may be thought of as a virtual one in absence of an H acceptor, but as a real one in presence of an H acceptor. .The hydrogen atoms are oxidized by molecular oxygen or dye but no cystine is produced. All of the more complicated complexes may be imagined to arise from the primary compound $(RS)_2Hg$ and to be in equilibrium with it. For convenience we shall hereafter simply denote the mercury-cysteine complexes by $Hg(RS)_2$.

Comparison of the reaction of cyanide and cysteine so far discussed shows them to be remarkably analogous and leads

directly to the same interpretation for the cysteine potential at mercury as is accepted for the cyanide potential at mercury:

$$Hg + 2HCN \rightleftharpoons Hg(CN)_2 + 2H$$
$$Hg + 2RSH \rightleftharpoons Hg(SR)_2 + 2H$$
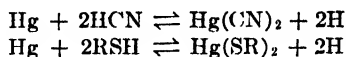
This interpretation was tested by an experiment equivalent to that used for cyanide. An electrode vessel containing an $M/15$ solution of Na_2HPO_4 was thoroughly bubbled with purified nitrogen; then cysteine was added. By means of an agar bridge saturated with KCl, this half-cell was connected with a calomel electrode and the circuit completed through a milliammeter which showed a steady current of 0.25 milliampere. A control experiment was made by preparing a vessel in exactly the manner described, except that there were no electrical connections. The experiments were discontinued after 6 hours. The solution of the half-cell through which current passed gave a heavy precipitate of HgS with H_2S. No mercury was demonstrable in the solution of the control experiment with open circuit. Hence it is evident that mercury in a cysteine solution works as an attackable electrode and not as an indifferent one.

The cysteine potential is then to be described by:

$$E = E_0 + \frac{RT}{F} \log [H^+] - \frac{RT}{F} \log \frac{[RSH]}{\sqrt{[Hg(SR)_2]}} \tag{3}$$

The formula found experimentally (1, 2) was:

$$E = E_0 + \frac{RT}{F} \log [H^+] - \frac{RT}{F} \log [RSH] \tag{4}$$

and it seems surprising perhaps that it was unnecessary to consider the concentration of $Hg(RS)_2$. This may be accounted for in the following way. In Paper I it was emphasized that strict regularity of experimental procedure was necessary for reproducible values. A buffer solution was added to a vessel containing a mercury electrode and only after this solution was freed from air by bubbling with nitrogen was cysteine added. Bubbling with nitrogen was then resumed. It is certain that a minute amount of mercury is oxidized before the air has been completely expelled from the solution. This oxidized mercury then combines with

the added cysteine. Experimental conditions were so strictly regular that the amount of oxidized mercury must have been approximately equal in all experiments, and consequently the concentration of $(RS)_2Hg$ approximately constant for all experiments of this kind.

Two sets of experiments have been done to test this argument. A 0.01 M solution of cysteine in standard acetate solution of pH 4.6 (iron is not a catalyst at this pH) was allowed to remain in contact with a mercury electrode in the presence of air for about half an hour at 37.5°, in contrast to the arrangement of the experiments just described. Air was then displaced by purified nitrogen. The potential was quickly established but was from 20 to 30 millivolts more positive than that previously reported (1). A saturated solution of crystals of $Hg(SR)_2$ was then made in standard acetate and 0.5 cc. of this solution added to the 10 cc. in the electrode vessel. The potential became more positive, but only by 5 millivolts.

In the other set of experiments cysteine was added to a buffer at a mercury electrode only after expulsion of oxygen by nitrogen. After establishment of the anticipated potential, mercurous nitrate was added to the solution from a burette. The following protocol is of such an experiment.

A standard acetate solution, 0.01 M with respect to cysteine, HCl and at a temperature of 37.5° is bubbled with purified nitrogen. A mercury electrode vessel is used. The mercurous nitrate added is 0.004 M with respect to mercury (by gravimetric analysis as HgS). A calomel electrode in 4 M KCl is used as reference electrode.

	Potential. $mv.$
No $HgNO_3$ yet added.....................................	−383
$HgNO_3$ added to give 0.000004 M Hg^+......................	−381
" " " " 0.000015 " "	−377
" " " " 0.00012 " "	−369
" " " " 0.00092 " "	−354

It appears surprising in such experiments that the potential depends so little on the concentration of mercury dissolved as complex. If the only complex present were $(RS)_2Hg$, then according to equation (3) we should expect a potential difference of 30 millivolts when the concentration of dissolved mercury is

changed ten times.   The variation of potential, however, is found
to be much less.   A theoretical treatment of this problem is as
difficult as with cyanide and the difficulty is due most probably to
formation of many complexes.   Addition of increasing quantities
of mercury salts to a cysteine solution not only increases the con-
centration of the simple compound $(RS)_2Hg$ but also gives rise to
formation of higher complexes.   The activity of the mercurous
ions in solution—that quantity governing the magnitude of the
potential—increases much less than proportionally to the total
amount of dissolved mercury.   We believe this to be an important
factor in the apparent constancy of the term $\sqrt{[(RS)_2Hg]}$ of
equation (3) when conditions are made equivalent in all experi-
ments, as was true for the experiments of Paper I of this series.

<div align="center">DISCUSSION.</div>

It has been shown that cysteine reacts in the same way as
cyanide with metallic mercury.   Both reactions belong to the
general type: $2XH + Hg \rightleftharpoons HgX_2 + 2H$.   The concentration
of cystine in a cysteine solution is consequently without influence
on the potential at a mercury electrode.   This is an observation
which in the past has been without explanation and which falsely
contributed to considering the "cysteine-cystine system" a new
type of redox system.

It was pointed out in Paper I (1) that rate of bubbling greatly
affects the potential in presence of low concentrations of oxygen.
At rest the potential is often 70 millivolts more positive than with
moderate bubbling.   This observation now finds an explanation.
At rest and in the presence of oxygen, that portion of the cysteine
solution at the mercury surface reacts to form mercuric cysteinate.
The concentration of cysteine in this part of the solution dimin-
ishes therefore and the concentration of cysteinate rises; the
potential consequently becomes more positive.   On agitation,
the solution at the mercury surface has no less cysteine nor more
cysteinate than the bulk of the liquid and the potential therefore
is more negative.   It is important, however, to agitate gently
the solution even when it is saturated with purified nitrogen;
this precaution, taken for an inadequate reason, was observed in
previously published measurements of the cysteine potential.

The deviation of the potential at pH's higher than 9.5 from the

values fixed by the equation valid for a lower pH range is to be related to the observation given above; one, at least, of the mercury-cysteine complexes formed at pH's higher than 9.5 is of a different composition and crystalline structure than is got at pH's lower than 9.5.

In conclusion it must be said that the values of the cysteine potential at mercury are not those necessary to characterize the reduction intensity of cysteine under physiological conditions. The mercury electrode is not an inert electrode to be used for measurement of an intrinsic property of cysteine but it is an attackable electrode and the potential is established by a chemical reaction between mercury and cysteine.

The problem immediately arises as to whether the behavior of the mercury electrode is the same as that of platinum and gold electrodes which are always considered truly indifferent electrodes. An answer to this problem is necessary for the correct interpretation of all reduction potentials measured in tissues or bacterial cultures by means of such "indifferent" electrodes. This interpretation is of such great importance that it justifies a separate study of platinum and gold which is soon to be reported.

CONCLUSIONS.

Cysteine reacts in the same way as cyanide with metallic mercury. Both form reducing systems with the metal consequent upon this sort of reaction:

$$2XH + Hg \rightleftharpoons HgX_2 + 2H$$

The correct formula for the cysteine potential at mercury is therefore:

$$E = E_0 - \frac{RT}{F} \ln \frac{[RSH]}{\sqrt{[(RS)_2Hg]}} + \frac{RT}{F} \ln [H^+]$$

The concentration of $(RS)_2Hg$ is so little altered by reaction between RSH and metallic mercury in the absence of oxygen, that it can be included in the constant of the equation when experimental results are evaluated.

It is improper to speak of a "cysteine-cystine system" at mercury. It is the "cysteine-mercuric cysteinate system" which

determines the potential. This system is not related to the problem of cysteine oxidation in metabolism.

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THE PREPARATION OF LYSINE, HISTIDINE, AND ARGININE FROM HYDROLYZED BLOOD CORPUSCLE PASTE BY ELECTRICAL TRANSPORT.

By GERALD J. COX, HARRIETTE KING, AND CLARENCE P. BERG.

(From the Laboratory of Physiological Chemistry, University of Illinois, Urbana.)

INTRODUCTION.

In seeking economical methods for the preparation of the amino acids, we have made use of the excellent procedure of Foster and Schmidt (1921–22, 1923, 1926; Ikeda and Suzuki, 1912) for the preparation of lysine, histidine, and arginine by electrical transport from hydrolyzed protein. This communication records our experience in the practical separation of the amino acids of hydrolyzed blood corpuscle paste. We have selected that starting material because it is readily available and yields large amounts of the basic amino acids, particularly lysine and histidine.

We have made the following modifications of the Foster and Schmidt apparatus and procedure.

Our three compartment cells are constructed of thoroughly paraffined white pine. The diaphragms between the compartments are of parchmentized paper, instead of linen coated with formaldehyde-treated gelatin. Cooling is effected by passing a stream of water through 10 mm. glass tubes in the form of a series of S-bends placed between the carbon electrodes and the diaphragms. It is not necessary to stir the center compartment or to regulate the pH of any of the compartments during the transport of the amino acids from the hydrolyzed protein. During the retreatment for the separation of histidine, we have added sulfuric acid to the *anode* compartment to increase the conductivity of the cell. From the solutions obtained after electrical transport we have separated arginine as the flavianate, lysine as the picrate, and histidine as

the double salt with mercuric sulfate. We have found that a somewhat higher yield of lysine and arginine may be obtained if the suspension of barium sulfate, which results from the neutralization of the sulfuric acid hydrolysate of the blood paste, is subjected directly to electrolysis. The yield of histidine is slightly lowered, however.

The use of parchmentized paper reduces considerably the electroendosmotic transport of water and the consequent flooding of the cathode compartment toward the end of the electrolysis. The electroendosmotic rate for parchmentized paper is distinctly less than for the membranes employed by Foster and Schmidt. Moreover, the paper may be obtained ready for use and it is more durable and cleaner than the gelatin diaphragms.

Foster and Schmidt passed a current of carbon dioxide into the cathode compartment to reduce the alkalinity. They also regulated the pH of the center compartment by the addition of barium hydroxide or sulfuric acid. We have found that the most basic components of the amino acid mixture, ammonia, arginine, and lysine, migrate to the cathode first and oppose the entrance of the other amino acids. This is in accord with the theory that the monoaminomonocarboxylic acids are ionized as acids under the conditions of alkalinity developed in the cathode compartment. Any such amino acids which diffuse into the cathode compartment during the early stages of the separation will return to the center as the current increases. We have effected the separation of lysine and arginine from histidine by opposing the migration of the latter with the alkalinity of the two more basic amino acids.

We have attempted to save the time, labor, and losses involved in the filtration and washing of barium sulfate, which is formed when the sulfuric acid used in the hydrolysis of the blood paste is neutralized with barium hydroxide, by submitting the thick suspension directly to electrical transport. This procedure also obviates reconcentration of the large volumes of filtrate. We have succeeded in securing slightly higher yields of arginine and lysine but the histidine yield is lowered and the leucine and tyrosine which may be obtained as by-products are lost unless the barium sulfate is filtered off after the electrolysis. A considerable amount of iron is carried to the cathode when this modification is used on hydrolyzed blood, but it is precipitated completely as the hydrox-

ide and may be filtered off.   Another minor objection to the direct electrolysis of the neutralized hydrolysate is the necessity of removing a thick mud from the cells.   We do not recommend this procedure.
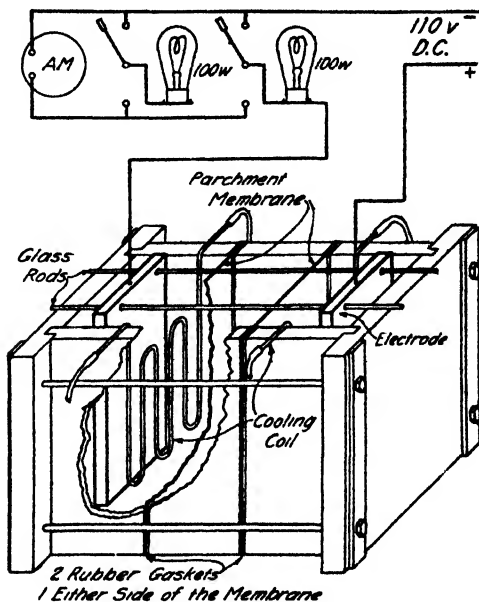
FIG. 1.   Three compartment electrolytic cell.

EXPERIMENTAL.

Hydrolyze 4 kilos (1 gallon) of blood corpuscle paste[1] by vigorous boiling under a reflux condenser for 30 hours with 3 liters of 50 per cent (by volume) commercial sulfuric acid.   After cooling,

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[1] This material was secured from Armour and Company, Chicago, according to the following specifications: "Fresh blood corpuscle paste, concentrated as much as possible and preserved well with toluene, for the preparation of histidine."   The yields of amino acids reported in this communication were obtained from paste that had 5.80 per cent of nitrogen and 36.2 per cent of dry material (obtained by drying to constant weight over sulfuric acid).   We advise analysis of the paste for either nitrogen or solids as a basis for use of reagents, particularly for avoiding an excess of picric acid in the preparation of lysine.

transfer the hydrolysate to a stoneware jar and dilute it with about 18 liters of water.   Add 8 kilos of solid barium hydroxide. Stir the mixture thoroughly for 30 minutes and complete the removal of the sulfuric acid by the careful addition of a solution of barium hydroxide.   Filter off the precipitate of barium sulfate on a 50 cm. stoneware filter or on 20 cm. Buchner funnels and wash it on the filter with three 5 liter portions of water.   Concentrate the filtrate and washings, *in vacuo*, to about 1500 cc., or until the separation of leucine and tyrosine makes further concentration difficult.   Cool the mixture in the ice box for 2 days.   Filter off the separated amino acids, wash with 200 cc. of ice water, and dry them in air.

The cell for the separation by electrical transport is constructed as follows (see Fig. 1):

A box 24 × 12 × 15 cm., inside dimensions, is constructed of white pine 2 cm. thick.   All joints are tongued and grooved. The ends are made to extend 3 cm. beyond the sides of the box. The box is sawed transversely into three equal sections and the pieces saturated with paraffin by immersion in the melted wax in a vacuum.   The box is reassembled as a three compartment cell by placing a sheet of parchmentized paper and two rubber gaskets at each section and clamping them together by means of four bolts passing through holes in the end extensions and along the sides of the box.   The wood is protected from the bolt heads by means of iron plates.   Graphite electrodes 16 × 11 × 1 cm. are placed in each end compartment and supported on two glass rods laid longitudinally on top of the cell and passed through small holes in the electrodes.   The direct current from a 110 volt line is led into the cell as shown in the wiring diagram.   By means of the double throw switch the ammeter may be thrown into or cut out of the circuit with any single cell.   The second 100 watt lamp in Fig. 1 represents the circuit for a cell not shown in the drawing.   Cooling of the cell is effected by passing water through 10 mm. glass coils placed between the electrodes and the membranes in the end compartments.

Place the combined filtrate and washings from the leucine and tyrosine in the center compartments of three electrolytic cells. Fill the end compartments with distilled water and subject the amino acids to electrical transport by a direct current of 110 volts

with a fuse plug in place of the lamp shown in Fig. 1. After the current reaches about 0.5 ampere place a 100 watt lamp in series with the cell. Continue the electrolysis for 12 hours. Remove the solution in the basic compartment, Fraction I, and replace it with distilled water. Continue the electrolysis, testing for histidine at hourly intervals with Hunter's (1922) test. When a strong positive test is obtained, remove the solution, Fraction II, from the basic compartment and again refill with distilled water. Electrolyze until the center compartment gives only a faint cloudiness when tested with phosphotungstic acid (12 to 15 hours). Discard the contents of the center and acidic compartments. The solution at the cathode, Fraction III, contains most of the histidine.

Only Fraction II obtained above needs to be reelectrolyzed. Combine the solutions from the three cells and concentrate under reduced pressure to about a liter. Place in the center compartment of a cell and electrolyze with distilled water in the cathode and approximately 0.5 per cent sulfuric acid in the anode compartment. Continue the electrolysis until a faintly positive test for histidine is obtained at the cathode. Combine the solution from the basic compartment with Fraction I obtained above, and the center compartment with Fraction III. Discard the anodic solution. Filter Fraction I to remove any ferric hydroxide and concentrate to about half its original volume to remove ammonia. Dilute it to 8 liters and make it acid to litmus with sulfuric acid. Precipitate the arginine by the addition of 100 gm. of flavianic acid (1-naphthol-2, 4-dinitro-7-sulfonic acid) dissolved in 500 cc. of water. Allow it to stand in the ice box for 24 hours, filter off the arginine flavianate, and wash it with ice water. Dry the precipitate and convert it to arginine monohydrochloride (Cox, 1928).

Add a hot, concentrated solution of barium hydroxide to the filtrate and washings from the arginine flavianate until no further precipitate of barium flavianate is obtained. Filter, wash the barium flavianate, and exactly remove the barium from the combined filtrate and washings by means of dilute sulfuric acid. Filter off the barium sulfate. Concentrate the filtrate, *in vacuo*, to a thin sirup. Dissolve the sirup in 500 cc. of 95 per cent ethyl alcohol and add, with stirring, 120 gm. of picric acid dissolved in 1200 cc. of alcohol. Place in the ice box overnight. Filter a por-

tion and test for complete precipitation by the addition of alcoholic picric acid. If a precipitate is obtained add more picric acid to the main solution and allow it to stand several hours in an ice bath. Avoid an excess of picric acid as the dipicrate of lysine is soluble in alcohol. Filter off the lysine picrate and wash it with two 100 cc. portions of alcohol. Dry the crystals and recrystallize by solution in the minimum amount of boiling water (about 10 cc. per gm.), filtration, and cooling in the ice box overnight. Filter off the lysine picrate, wash with ice water, and dry in air. More picrate may be secured from the aqueous mother liquor, but it must be recrystallized.

Transfer the lysine picrate to a 2 liter flask, add 300 cc. of water and 300 cc. of concentrated hydrochloric acid. Heat the mixture on a steam cone for 30 minutes with frequent stirring and remove the picric acid by extraction in a separatory funnel once with 400 cc. and three times with 100 cc. portions of hot benzene. Add 25 gm. of norit to the lysine solution, boil several minutes, and filter into a 3 liter flask. If the filtrate is not colorless, repeat the treatment with norit. Concentrate the solution to a sirup, *in vacuo*, and dissolve it by warming in the minimum amount (300 to 500 cc.) of 95 per cent alcohol. Cool the solution and add ethyl ether in 50 cc. portions, with shaking, until a permanent turbidity is obtained. Scratch the sides of the flask with a glass rod until definite crystals are formed. Add ether, 100 to 200 cc. at a time, with vigorous stirring and shaking, until the total volume added is 5 times the volume of alcohol used. It is essential that the ether be added in small enough portions that a thoroughly crystalline precipitation can be maintained. The formation of an oily precipitate will result in hygroscopic lysine dihydrochloride. When all the ether has been added, set the flask in the ice box overnight. Filter by suction and wash with small portions of ether. Dry in air or in a vacuum oven at 60°.

Recrystallize the lysine dihydrochloride by dissolving it in the minimum amount of hot alcohol, cooling, and adding ether. The same precautions as to stirring and adding the ether must be observed as in the first crystallization.

Filter off any precipitate of ferric hydroxide that may have separated from Fraction III and concentrate to a volume of about 1800 cc. Add 200 cc. of dilute (50 per cent by volume) sulfuric

acid and precipitate the histidine with 400 gm. of mercuric sulfate dissolved in 4 liters of 5 per cent by volume sulfuric acid. Allow the mixture to stand for 2 days to complete the precipitation. Filter and wash the precipitate with 1500 cc. of a 1 per cent solution of mercuric sulfate in 5 per cent sulfuric acid. Complete the washing with 300 cc. of water. Suspend the precipitate in 1 liter of water and add 100 cc. of 37 per cent hydrochloric acid. The histidine double salt is not completely dissolved by this amount of acid but a gummy precipitate forms if more is added. Completely remove the mercury by shaking with hydrogen sulfide. Filter off the mercuric sulfide and wash it with water until the washings are chloride-free. Exactly remove the sulfuric acid from the filtrate by the addition of a concentrated solution of barium hydroxide. Filter off the barium sulfate. The filtrate should be practically colorless. Concentrate under reduced pressure *in a weighed flask* to a thick sirup. Continue heating the flask, *in vacuo*, in a boiling water bath for 1 hour. Add a volume of 37 per cent hydrochloric acid equal to the weight of the residue in gm. Heat on the steam cone, with stirring, until the crude crystals and sirup are completely dissolved. Cool the solution, seed with histidine dihydrochloride, if necessary, and scratch the sides of the flask with a glass rod until crystals are formed. Place the flask in the ice box for 2 days to complete the crystallization. Filter off the histidine dihydrochloride and dry it in the air or in a vacuum oven.

Dissolve the histidine dihydrochloride in 500 cc. of water, add 10 gm. of norit, boil several minutes, and filter. Concentrate the filtrate, *in vacuo*, to a thin sirup.   Dissolve the histidine dihydrochloride by heating in 500 cc. of 95 per cent alcohol and precipitate histidine monohydrochloride by adding 50 cc. of aniline with vigorous stirring. Cool overnight in an ice box. Filter and wash with alcohol. The histidine monohydrochloride must be recrystallized to remove traces of aniline which tend to discolor the product on long standing. Dissolve in the minimum amount of hot water, filter if necessary, and precipitate the histidine chloride by the addition of 500 cc. of alcohol. Filter off the crystals and dry in air or in a vacuum oven.

To separate leucine and tyrosine from the crude precipitate which separates from the original hydrolysate, boil the dry mixture

for 15 minutes under a reflux condenser with 8 cc. of glacial acetic acid for every gm. of solid. Filter hot and extract the residue of tyrosine with 200 cc. of boiling glacial acetic acid. Place the filtrate and washings in the ice box for 24 hours. Filter off the leucine which crystallizes out of the acetic acid and evaporate the filtrate to dryness under reduced pressure. Recrystallize the residue and the crystallized leucine separately by dissolving in a minimum of boiling water, treating with 25 gm. of norit, filtering, and cooling in the ice box overnight. Filter, and wash with ice water. Dry in the air. A considerable portion of the leucine remains in solution and may be recovered by concentrating, *in vacuo*, to a small volume and cooling. The leucine thus obtained must be recrystallized.

The residue of crude tyrosine remaining after extraction of leucine with glacial acetic acid is purified by dissolving in 500 cc. of water containing 10 gm. of sodium hydroxide. Boil the solution and decolorize with 10 gm. of norit. Filter, and precipitate the tyrosine by adding 37 per cent hydrochloric acid until crystallization begins. Add glacial acetic acid until the mixture is acid to litmus. Mix thoroughly and place in the ice box overnight. Filter and wash the tyrosine with ice water until the washings are chloride-free. Dry in air or in a vacuum oven.

The amino acids as obtained above have been analyzed repeatedly for amino nitrogen with uniformly satisfactory results. The yields of the *pure* final products from 4 kilos of blood corpuscle paste are as follows:

		gm.
l-Histidine monohydrochloride, $C_6H_9N_3O_2 \cdot HCl + H_2O$		51
d-Arginine " $C_6H_{14}N_4O_2 \cdot HCl$		21
d-Lysine dihydrochloride, $C_6H_{14}N_2O_2 \cdot 2HCl$		62
l-Leucine, $C_6H_{13}NO_2$		58
l-Tyrosine, $C_9H_{11}NO_3$		22

DISCUSSION.

Histidine.—Instead of mercuric chloride and sodium carbonate we have used mercuric sulfate in sulfuric acid to precipitate histidine because (1) it is a more specific precipitant, (2) smaller volumes are encountered, and (3) the precipitate is more readily

filtered and washed.   Precipitation is complete in 2 days, as shown by the Hunter (1922) test on the filtrate.

We have chosen histidine monohydrochloride as the best final form for that amino acid because of the high yields that may be obtained in purification.   Conversion of the dihydrochloride to the monohydrochloride, including the purification by recrystallization, gave 89 per cent of the theoretical.   The method is the same as that used by one of us (Cox, 1928) in the preparation of arginine monohydrochloride.   The dissociation constants of aniline and histidine given by Michaelis (1922) are as follows:

Aniline .... K_b = 4.6 × 10^{-10}
Histidine.................................. K_{b_1} = 5.7 × 10^{-9}
                                            K_{b_2} = 5.0 × 10^{-13}

The corrected melting point of our histidine monohydrochloride is 259°.   Abderhalden and Einbeck (1909) report 255°.

The success of electrical transport for the preparation of histidine may be judged by comparison with two different methods which use blood corpuscles as starting material.   Hanke and Koessler (1920) precipitate histidine by means of mercuric chloride and sodium carbonate and report a yield about double that obtained by our procedure.   Using their method we have never been able to secure more than half the yield they claim.   Vickery and Leavenworth (1928), employing silver oxide to precipitate histidine, secure about 20 per cent more of the amino acid than we have obtained, if the yields are calculated on the basis of the nitrogen of our respective starting materials.   They used a partially purified hemoglobin which is obviously richer in histidine than our crude corpuscle paste.

Arginine.—We have found it advisable to modify in one detail the method of preparing arginine monohydrochloride from the flavianate.   If the sirup of arginine dihydrochloride is dissolved in alcohol and precipitated with aniline, a dark colored product is thrown down which may not crystallize readily.   Also if the sirup is not concentrated sufficiently, considerable more aniline is needed to neutralize the excess hydrochloric acid.   We have, therefore, dissolved the sirup in water, allowed the small amount of flavianic acid to separate as arginine flavianate, decolorized with norit, evaporated to dryness, and proceeded then with the alcoholic

treatment.   The corrected melting point of the arginine monohydrochloride obtained is 222°.

Lysine.—Our lysine dihydrochloride melts at 200–201°, corrected.   Henderson (1900) and Szydlowski (1906) report 192–193°.   Our product is non-hygroscopic if the crystallization process is properly conducted.

Several attempts to prepare lysine monohydrochloride by the use of aniline yielded a material of composition intermediate between the mono- and the di-salt.   Recrystallization by various procedures or retreatment with aniline did not result in a pure monohydrochloride.

We believe the method of electrical transport to be by far the most practical method for the preparation of lysine in quantity as it obviates entirely the use of large amounts of silver salts and phosphotungstic acid and also the labor involved in the manipulation and recovery of these reagents.

Leucine and Tyrosine.—Larger yields of leucine can be obtained from blood paste by the use of more intensive methods than we have employed.   We have not attempted to secure these higher yields as we were primarily interested in the basic amino acids. Ehrlich and Wendel (1908) report $[\alpha]_D^{20} = + 15.6°$ for leucine in 20 per cent hydrochloric acid.   We have found values from $+14.2°$ to $+15.5°$ for our preparations.

The tyrosine is readily obtained in fairly constant yields.

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SARMENTOCYMARIN AND SARMENTOGENIN.

BY WALTER A. JACOBS AND MICHAEL HEIDELBERGER.

(From the Laboratories of The Rockefeller Institute for Medical Research, New York.)

The present communication owes its origin to a circumstance which attended our first efforts some years ago (1915) in the investigation of strophanthin. The first specimen of *Strophanthus* seeds (20 pounds), which had been purchased from a commercial source as *Strophanthus hispidus* seeds, yielded after extraction and hydrolysis a "genin" (31 gm.) which differed analytically and in other respects from the strophanthidin recorded as occurring in the active glucosides of both *kombe* and *hispidus* seeds. Unfortunately, without anticipating such a complication, no samples of these first seeds were retained so that subsequent botanical identification was impossible. All later samples of seeds which were obtained from commercial sources were in the main either *Strophanthus hispidus* or *Strophanthus kombe* and the aglucone obtained from them by the usual procedure proved to be the already known strophanthidin, except that the mother liquors occasionally yielded very small amounts of the first aglucone. But to trace its source in such commercial mixtures of seeds was a hopeless undertaking.

The attempt was then made to obtain directly from Africa, which is the source of all commercial *Strophanthus* seeds, samples of different varieties that had been properly identified. In this undertaking we were very fortunate to obtain the generous cooperation of Dr. Wilbur A. Sawyer of the International Health Division of the Rockefeller Foundation and of officials connected with the Lands and Forests Department at Freetown, Sierra Leone, West Africa. Through their efforts we have received samples of *Strophanthus hispidus, Strophanthus sarmentosus,* and *Strophanthus gratus.* In addition small quantities of *Strophan-*

thus courmontii and *Strophanthus eminii* were received from similar departments in Nyasaland Protectorate and Tanganyika Territory of East Africa respectively.[1]

The results of the study of *Strophanthus hispidus* have already been published.[2] The samples of *courmontii* and *sarmentosus* proved on investigation to contain only traces of bitter glucosides which could be salted out with ammonium sulfate and amounts too small for study. The low glucoside content in these cases may possibly be referable to differences among individual plants of the same species or perhaps to seasonal influences. On the other hand the sample of *Strophanthus eminii* was found to be fairly rich in a bitter glucoside which, however, resisted our preliminary attempts at successful investigation. More recently the study of this material has been resumed and it is hoped to present the facts obtained at a later time.

We have, however, been fortunate enough to obtain from another source a *Strophanthus* seed which has made possible a provisional conclusion to our earliest studies. This material was most generously presented to us several years ago by Dr. J. C. Munch, at that time connected with the Pharmacological Laboratory of the Bureau of Chemistry, now called the Food, Drug and Insecticide Administration, United States Department of Agriculture at Washington. According to Dr. Munch this *Strophanthus* seed had been originally identified as *Strophanthus sarmentosus*. In the hands of several pharmacognists this identification appears to have been verified. The pharmacognostic findings will be published by Munch and coworkers independently.

Our first attempts to obtain a crystalline aglucone directly by the usual method from the purified extract of these seeds were unpromising. This was caused by the presence of an unusual amount of saponins and other substances which interfered with crystallization. More recently this material was found to yield

to the same method of investigation which has been successfully used in the study of *kombe*[3] and *hispidus* seeds.[2] A crystalline glucoside has been obtained, which on hydrolysis yielded an aglucone identical in all respects with that obtained at the beginning of our studies. This does not permit any conclusion as to the identity of the seeds used in the early work since it has already been the experience that the same glucosides may occur in different species of *Strophanthus* seeds. The present chemical studies, however, made partly with the aglucone of undetermined origin and in part with the identical substance occurring in the seeds identified as *Strophanthus sarmentosus* are presented not so much as a contribution to the chemistry of any particular botanical species but rather as an interesting addition to our previous reports on the chemistry of the cardiac aglucones. Finally, we have been privately informed by Dr. Munch that in a preliminary study of these seeds at the Bureau of Chemistry they were successful in obtaining small amounts of a crystalline substance which had the same toxicity for frogs as ouabain.

The purified aqueous solution obtained from the alcoholic extract of the Washington *sarmentosus* seeds, when extracted with chloroform gave small amounts of a substance which was finally obtained in beautifully crystalline form. This substance proved to be a glucoside and very closely allied in chemical properties to cymarin and periplocymarin.[4] The analytical figures agreed best with the formulation $C_{30}H_{46}O_8$. The substance, which we have called *sarmentocymarin*, is therefore isomeric with periplocymarin. Like the latter and cymarin, it gives the Keller-Kiliani reaction characteristic of α-desoxy sugars. It also contains one methoxyl group which was found to belong to the sugar.

The mixture of glucosides which remained in the very bitter aqueous solution following the chloroform extraction was found to yield to the same treatment which was employed in the case of *kombe* strophanthin and periplocin. By digestion with an enzyme which was prepared from fresh *Strophanthus sarmentosus* seeds (obtained from West Africa) a cleavage of sugar occurred and it became possible again to extract with chloroform a considerably

[3] Jacobs, W. A., and Hoffmann, A., *J. Biol. Chem.*, **69**, 153 (1926).

[4] Jacobs, W. A., and Hoffmann, A., *J. Biol. Chem.*, **79**, 519 (1928).

larger amount of glucoside identical with the sarmentocymarin described above. The sugar which was cleaved during this hydrolysis was readily identified as d-glucose. The water-soluble, chloroform-insoluble glucosides of *Strophanthus sarmentosus* are therefore glucosido- or perhaps polyglucosidosarmentocymarins in which a glucose molecule is bound on the desoxy sugar as previously demonstrated in the case of the *kombe* and *hispidus* strophanthins and periplocin.

Sarmentocymarin exhibited the lability towards acids characteristic of the glucosides of α-desoxy sugars and was readily hydrolyzed to the crystalline aglucone, *sarmentogenin*, and a methyl ether desoxy sugar. Up to the present, the latter has been obtained only as a syrup and although it must possess the same formulation as cymarose, attempts to induce its crystallization by seeding with the latter have been unsuccessful.

Sarmentogenin on analysis gave figures compatible with the above formulation of sarmentocymarin and possesses the formula $C_{23}H_{34}O_5$. It is isomeric with both periplogenin and gitoxigenin. In other respects it possesses the chemical characteristics of the strophanthidin group of cardiac aglucones. Although neutral, it contains a lactone group which was readily ascertained in saponification experiments. On catalytic hydrogenation, one double bond was demonstrated by the formation of a *dihydro* derivative. Sarmentogenin also exhibits the behavior characteristic of a $\Delta^{\beta,\gamma}$-lactone. It gives a positive nitroprusside reaction, a property which is lost on conversion to dihydrosarmentogenin. Like gitoxigenin and periplogenin, it possesses three hydroxyl groups. The formation of a monoketone, *sarmentogenon*, on oxidation has shown one of these groups to be secondary. Two of the hydroxyl groups may be acylated with the formation of a *dibenzoate*. Since this derivative was resistant to chromic acid, the secondary hydroxyl group must have been involved in the acylation.

The behavior of sarmentogenin towards alkali has given additional information in regard to its structure. Under the special conditions described in the *Experimental* part, it was isomerized to *isosarmentogenin* in a manner analogous to the formation of iso compounds from strophanthidin, digitoxigenin, and periplogenin. This iso compound, like the parent substance, forms a *dibenzoate*.

Like the other iso compounds, isosarmentogenin no longer

gives the nitroprusside reaction and cannot be hydrogenated. On saponification it yields an acid which shows the characteristic behavior of these substances towards hypobromite. Oxidation readily occurred with the formation of a lactone acid, *isosarmentogenic acid*, which from analysis and titration was shown to possess the formula $C_{22}H_{34}O_6$. This was confirmed by the study of its beautifully crystallizing methyl ester.

Since these observations parallel exactly our experience with isostrophanthidin,[5] the following conclusions may be drawn.

Isosarmentogenin is a lactone of the lactol form of a hydroxyaldehyde. On saponification a hydroxyaldehydo acid results which may exist in either the free aldehydic or lactol form. In this acid the aldehydic carbon is γ to the carboxyl group and a hydroxyl group of tertiary character is attached to a carbon which is in turn presumably γ to the aldehydic carbon.

These transformations, as in the case of isostrophanthidin and other iso compounds, may be represented graphically as follows:

$$
\begin{array}{ccc}
\text{CH}_2\!\!-\!\!-\!\!-\!\!\text{C} & \!\!=\!\!=\!\! & \text{C} \\
| & | & | \\
\text{CO} & \text{CH}_2 & \text{HOC} \\
& \diagdown\!\text{O}\!\diagup &
\end{array}
\qquad
\begin{array}{ccc}
\text{CH}_2\!\!-\!\!-\!\!\text{CH}\!\!-\!\!-\!\!\text{CH} \\
| \qquad | \qquad | \\
\text{CO} \quad \text{CH} \quad \text{C} \\
\diagdown\!\text{O}\!\diagup \; \diagdown\!\text{O}\!\diagup
\end{array}
$$
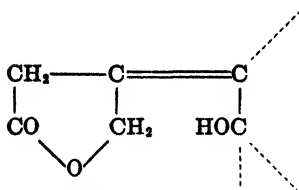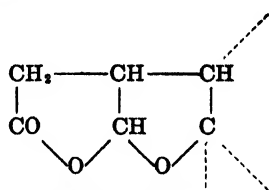
Sarmentogenin.   Isosarmentogenin.

$$
\begin{array}{ccc}
\text{CH}_2\!\!-\!\!-\!\!-\!\!\text{CH}\!\!-\!\!-\!\!\text{CH} \\
| \qquad\qquad | \qquad | \\
\text{COOH} \quad \text{HOCH} \quad \text{C} \\
\diagdown\!\text{O}\!\diagup
\end{array}
\qquad
\begin{array}{ccc}
\text{CH}_2\!\!-\!\!-\!\!-\!\!\text{CH}\!\!-\!\!-\!\!\text{CH} \\
| \qquad\qquad | \qquad | \\
\text{COOH} \quad \text{CO} \quad \text{C} \\
\diagdown\!\text{O}\!\diagup
\end{array}
$$
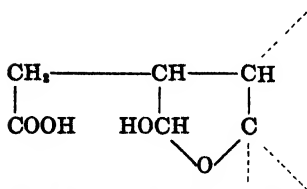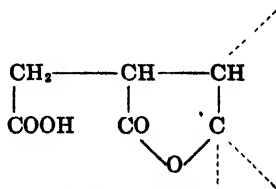
Isosarmentogeninic acid.   Isosarmentogenic acid.

Sarmentogenin is, therefore, a trihydroxy-$\Delta^{\beta,\gamma}$-lactone possessing 23 carbon atoms and four saturated rings.

In connection with the observations which we have just described, it is of interest to reconsider certain data which were

[5] Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **74**, 811 (1927).

presented by Kohn and Kulisch[6] 30 years ago in a communication on strophanthin supposedly obtained from *Strophanthus kombe* seeds.   In an excellent critical review of the chemical work on strophanthin which antedated their own (1912), Heffter and Sachs[7] definitely concluded that the strophanthin of Kohn and Kulisch was a substance of uncertain origin, distinctly different from the strophanthins of *kombe* and *hispidus* seeds and one which no other worker in the field of strophanthin had found again. From the analytical values reported by Kohn and Kulisch, it is apparent that their strophanthin was a polysaccharide compound, exactly as we have more recently shown to be the case with the amorphous *kombe* and *hispidus* strophanthins.   In the case of their aglucone, "strophanthidin," the average values which were found for C and H (71.07 and 8.63 respectively) are in close agreement with those which we have obtained with sarmentogenin and which are required by a $C_{23}H_{34}O_5$ formula.

A serious discrepancy is to be found in their report that the aglucone contained methoxyl, but the value given (5.6 per cent) is not compatible with any probable formulation for their aglucone which might be deduced from the analytical figures.   It is to be doubted that this result can be accepted, especially since no carefully studied aglucone of the cardiac glucosides has been found to contain alkoxyl.   In some cases forms have been obtained which retain solvent rather tenaciously.   The values for C and H and methoxyl obtained with their strophanthin appear quite compatible with those for a diglucosidomethyl ether desoside of a $C_{23}H_{34}O_5$ genin.   It is therefore not improbable that the "strophanthidin" which these workers had in hand was sarmentogenin. There is still a discrepancy in the melting point reported by them (193°) with that which we have observed in the case of sarmentogenin (265°).   The latter, however, when first obtained was found to exhibit considerable variation due possibly to dimorphism.   At any rate, the substance of Kohn and Kulisch, if not identical, was beyond question at least isomeric with sarmentogenin.

The above considerations make manifest the desirability of further extension of the study of the chemistry of the glucosides

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[6] Kohn, L., and Kulisch, V., *Monatsh. Chem.*, **19**, 385 (1898).

[7] Heffter, A., and Sachs, F., *Biochem. Z.*, **40**, 83 (1912).

which may be found in the properly identified plants belonging to or related to the *Strophanthus* family. The substances of the group already carefully studied have been shown to be very closely related in chemical structure as well as in pharmacological behavior. It is unfortunate that the plants in question are mostly rare and of tropical origin.

<div align="center">EXPERIMENTAL.</div>

Sarmentocymarin.—2500 gm. of *Strophanthus sarmentosus*, which had been obtained from Washington, were ground and defatted with gasoline. The dried, extremely bitter tasting powder was extracted three times with several volumes of 70 per cent alcohol. In each case the extract was sucked off thoroughly, followed by washings with the same solvent. The combined filtrates were precipitated in the usual manner with basic lead acetate. The copious precipitate was allowed to settle and finally was centrifuged off. The excess lead was removed from the supernatant liquid by careful addition of ammonium sulfate solution. The clear light brown solution was then concentrated under diminished pressure to about 1 liter, a process which was made very difficult when once the alcohol had been removed by the pronounced foaming caused by the accompanying saponins. When the aqueous solution was extracted with chloroform an intermediate layer of a thick gum containing chloroform at first formed. This intermediate gum was separated and separately extracted several times with chloroform. This extract was then added to the main chloroform solution which after washing with a few cc. of water was dried and concentrated to small bulk. When poured into petrolic ether a copious, amorphous precipitate was formed. After collection this precipitate weighed 5.1 gm.

This material was dissolved in dry acetone, filtered from small amounts of a sparingly soluble, voluminous impurity and concentrated to about 10 cc. When the solution was carefully diluted to a point just avoiding the permanent separation of an oily contamination and seeded, the glucoside slowly separated as a hard crust of stout, wedge-shaped prisms. These were collected and washed carefully with dilute acetone to remove the oily impurities. The substance was recrystallized by careful dilution of its concentrated alcoholic solution. It again separated as glisten-

ing, stout, pyramided prisms. From a warm concentrated solution in acetone, it was obtained in beautifully formed, broad, stout prisms which were 1 cm. long. From dilute alcohol or dilute acetone, the substance crystallized regularly with 2 mols of water of crystallization which were readily removed at 100° and 15 mm. Contrary to the experience with cymarin and periplocymarin, it could not be obtained from methyl alcohol in a form containing methyl alcohol of crystallization.

The hydrate melts slowly with effervescence at 130°. It is very sparingly soluble in water and is practically tasteless.

$$[\alpha]_D^{20} = -12.5° \text{ (c = 1.043 in methyl alcohol for the hydrate.)}$$

For analysis the substance was dried at 100° and 30 mm.

Air-Dry Substance.
21.440 mg. substance: 1.400 mg. H_2O.
    $C_{30}H_{46}O_8 \cdot 2H_2O$. Calculated. H_2O 6.31. Found. H_2O 6.53.
Anhydrous Substance.
4.075 mg. substance: 3.215 mg. H_2O, 10.022 mg. CO_2.
3.664 "   "   : 2.865 "   "   9.025 "   "
4.610 "   "   : 2.040 "   AgI.
        $C_{30}H_{46}O_8$. Calculated. C 67.37, H 8.68, OCH_3 5.80.
                        Found.   " 67.06, " 8.83.
                        "   " 67.18, " 8.75.
                        " OCH_3 5.84.

The original aqueous fraction obtained after removal of the chloroform-soluble glucoside was combined with the intermediate gummy layer and the adhering chloroform was removed from the mixture by suction. The resulting solution when saturated with ammonium sulfate deposited a copious, gummy precipitate. After decantation this was redissolved in water and again salted out with ammonium sulfate. The purified gum was dissolved in 95 per cent alcohol and the solution was filtered from ammonium sulfate. After concentration of the filtrate and washings to dryness under diminished pressure, the brown residual gum was dissolved in 1000 cc. of water. Attempts to obtain crystalline material from this solution or to obtain a crystalline aglucone directly by hydrolysis were unsuccessful. This was due apparently to the preponderating amounts of other substances in the mixture. The solution, which contained but negligible amounts of reducing

sugar, was then treated with 2 gm. of an enzyme which had been prepared from *Strophanthus sarmentosus* seeds of West African origin. After addition of toluene the mixture was placed in a thermostat at 38°.[8]

After 7 days the reducing power of the solution appeared to have reached a maximum, and appeared equivalent to about 10 gm. of free sugar calculated as glucose. The solution was treated with 5 volumes of alcohol in order to coagulate the enzyme. After concentration of the filtrate to remove the alcohol, the aqueous solution was extracted repeatedly with chloroform. The separation of the solutions rendered difficult by a gummy precipitate was facilitated by centrifuging. The concentrated chloroform extract upon being precipitated by petrolic ether gave a voluminous amorphous precipitate which was collected with petrolic ether. The yield was 21 gm. This was obtained in crystalline form by solution in a small volume of alcohol and careful dilution. When seeded, a copious crystallization of characteristic short, stout, pointed prisms occurred, which proved to be identical in all respects with the previously described glucoside. After recrystallization from dilute alcohol, it melted and effervesced at 130°.

$$[\alpha]_D^{20} = -12.0 \ (c = 0.994 \text{ in methyl alcohol for the hydrate.})$$

Air-Dry Substance.—Dried at 100° and 30 mm.

41.640 mg. substance: 2.780 mg. H_2O.

$C_{30}H_{46}O_8 \cdot 2H_2O$. Calculated. H_2O 6.31. Found. H_2O 6.68.

Anhydrous Substance.

3.485 mg. substance: 2.740 mg. H_2O, 8.575 mg. CO_2.

4.835 " " : 3.778 " " 11.895 " "

4.242 " " : 1.840 " AgI.

$\quad\quad C_{30}H_{46}O_8$. Calculated. C 67.37, H 8.68, OCH_3 5.80.

$\quad\quad\quad\quad\quad\quad$ Found. " 67.10, " 8.80.

$\quad\quad\quad\quad\quad\quad\quad$ " " 67.09, " 8.75.

$\quad\quad\quad\quad\quad\quad\quad$ " OCH_3 5.72.

After removal of adhering chloroform from the aqueous solution containing the sugar which had been cleaved by the enzyme,[9] it was saturated with ammonium sulfate to precipitate the complex substances of undetermined nature which still preponderated in

[8] In preliminary experiments the use of chloroform as a preservative was found inadvisable since the enzyme rapidly lost its activity.

the mixture. The unpleasant, slimy precipitate was separated by centrifuging. The clear solution was then precipitated with 4 volumes of alcohol. The filtrate from the solid·ammonium sulfate was concentrated to about 100 cc. and again treated with 4 volumes of alcohol in order to remove the remaining ammonium sulfate. The filtrate on concentration gave a residue of crude sugar which was dissolved in water. The resulting solution was found to reduce Fehling's solution to an extent corresponding to 38.8 mg. per cc. The rotation of the solution in a 1 dm. tube was found to be

$$\alpha_D = +1.98° \text{ or } [\alpha]_D = +51 \ (c = 3.88). \text{ For } d\text{-glucose } [\alpha]_D = +52.5°.$$

That the sugar was, therefore, glucose was confirmed by the preparation of an osazone which after recrystallization from 70 per cent alcohol melted at 210°. In its optical behavior, the osazone agreed in all properties with d-glucosazone. A solution of 0.1010 gm. in a mixture of 2 cc. of pyridine and 3 cc. of alcohol showed an initial rotation of $\alpha_D = -0.67°$ in a 0.5 dm. tube. After 24 hours $\alpha_D = -0.39°$.

4.790 mg. substance: 0.676 cc. N (26°, 751 mm.).
$C_{18}H_{22}O_4N_4$. Calculated. N 15.64. Found. ˙ 15.94.

Sarmentogenin.—A solution of 1.95 gm. of sarmentocymarin in 20 cc. of alcohol was treated with 40 cc. of 10 per cent hydrochloric acid. After 6 hours at room temperature the aglucone slowly crystallized when rubbed. The process was completed by further standing at low temperature after dilution. The collected substance weighed 1.2 gm. An additional 0.1 gm. was obtained from the concentrated filtrate after successive treatment with Ag_2CO_3 and H_2S to remove the Cl ions.

When recrystallized from dilute alcohol, it separates either as minute, short, stout prisms or six-sided platelets, depending upon the conditions. When it is recrystallized from 85 per cent alcohol, it forms a crust of prisms which are anhydrous and melt at 265–266°. On occasions the "genin" was obtained in a form which contained solvent of crystallization and melted at 243°. The anhydrous high melting form was most frequently encountered. It is appreciably soluble in warm methyl and ethyl (95 per cent) alcohols and but sparingly so in acetone and chloroform. It is practically insoluble in benzene and ether. Although the dry

powder is practically tasteless, the dust causes irritation and the gradual development of a bitter taste in the nasopharynx. The substance in dilute pyridine solution gives a deep red color with alkaline nitroprusside solution.

In its behavior towards concentrated sulfuric acid it closely resembles periplogenin. When covered on a watch-glass with the acid, it dissolves with a bright golden color and slowly develops a greenish color around the edges, which deepens to an indigo. This color after several hours extends throughout the solution. Strophanthidin does not develop an indigo color.

$$[\alpha]_D^{20} = +21.5 \ (c = 0.515 \ in \ 95 \ per \ cent \ alcohol.)$$

The substance does not contain methoxyl.

4.515 mg. substance: 3.635 mg. H_2O, 11.685 mg. CO_2.
4.080 "   "   : 3.260 "   "   10.560 "   "
$C_{23}H_{34}O_5$. Calculated. C 70.72, H 8.78.
Found.   " 70.57, " 9.00.
"   " 70.58, " 8.94.

When it is recrystallized from pyridine, it forms long micro platelets which melt at 258° with effervescence and contain approximately 1 mol of solvent. The latter adheres tenaciously.

7.600 mg. substance: 0.219 cc. N (21°, 747 mm.).
$C_{23}H_{34}O_5 \cdot C_5H_5N$. Calculated. N 2.98. Found. N 3.30.

Sarmentogenin Dibenzoate.—Benzoylation was readily accomplished in the usual manner in pyridine solution with benzoyl chloride. When the product was recrystallized by careful dilution of the warm acetone solution, it formed flat, hexagonal prisms which melted as 281°. It is very sparingly soluble in alcohol and practically insoluble in ether. The substance is not affected by chromic acid in acetic acid solution. The secondary alcoholic group of the "genin" is, therefore, involved in the benzoylation.

$$[\alpha]_D^{20} = +14. \ (c = 1.000 \ in \ acetone.)$$

3.646 mg. substance: 2.350 mg. H_2O, 9.896 mg. CO_2.
4.424 "   "   : 2.886 "   "   12.024 "   "
$C_{37}H_{42}O_7$. Calculated. C 74.21, H 7.08.
Found.   " 74.01, " 7.20.
"   " 74.11, " 7.29.

Dihydrosarmentogenin.—On catalytic hydrogenation 0.5 gm. of the genin suspended in methyl alcohol with 0.2 gm. of platinum

black slowly dissolved with simultaneous absorption of 30 cc. of
H_2 (1 mol). On evaporation of the solvent, the crystalline dihy-
dro compound remained. It separates from its concentrated
solution in 95 per cent alcohol as rather sparingly soluble, short,
broad prisms or plates which contain 1 mol of solvent of crystal-
lization and effervesce at 142°. It is readily soluble in acetone.
The substance does not give the Legal reaction.

Air-Dry Substance.
6.397 mg. substance: 2.993 mg. AgI.
$C_{23}H_{36}O_5 \cdot C_2H_5OH$. Calculated. OC_2H_5 10.26. Found. OC_2H_5 9.00.
Anhydrous Substance.—Dried at 100° and 30 mm.
4.035 mg. substance: 3.387 mg. H_2O, 10.440 mg. CO_2.
3.878   "   "   : 3.150 "   " 10.035 "   "
   $C_{23}H_{36}O_5$. Calculated. C 70.36, H 9.25.
     Found. " 70.56, " 9.38.
       "   " 70.57, " 9.09.

Sarmentogenon.—0.1 gm. of sarmentogenin was dissolved by
warming in a mixture of 2 cc. of acetic acid and 0.5 cc. of H_2O.
After rapid cooling an excess (0.4 cc.) of Kiliani chromic acid
solution was added. The reaction was rapid and leaflets soon
separated. These redissolved on dilution with water. On adding
saturated ammonium sulfate solution to turbidity, crystallization
of the ketone was facilitated. It forms glistening prismatic
needles from dilute alcohol, which melt at 226° and contain no
solvent. It is appreciably soluble in methyl and ethyl alcohols,
acetone, and chloroform.

When placed on the tongue, the substance develops an in-
tensely bitter taste which is far more marked than in the case of
the parent genin.

3.740 mg. substance: 2.720 mg. H_2O, 9.732 mg. CO_2.
   $C_{23}H_{32}O_5$. Calculated. C 71.08, H 8.31.
     Found. " 70.96, " 8.12.

Sarmentogenon Semicarbazone.—A solution of 0.1 gm. of the
ketone in 10 cc. of alcohol was treated with a mixture of 0.2 gm.
of semicarbazide hydrochloride and 0.3 gm. of potassium acetate
in 2 cc. of H_2O. After several days at room temperature, the
filtered solution was partly concentrated and carefully diluted.
Crystallization began after several days and the substance finally
separated as long, narrow platelets. Recrystallization was made
difficult by its sparing solubility and slow crystallization from

dilute solution. It was therefore dissolved in a relatively large volume of 85 per cent alcohol and concentrated to small volume. By careful dilution and further concentration, the solution slowly deposited compact aggregates of microscopic leaflets which melted at 200° with slow effervescence. The derivative is very bitter like the parent ketone.

For analysis the substance was dried at 100° and 30 mm.

5.125 mg. substance: 3.534 mg. H_2O, 12.150 mg. CO_2.
$C_{24}H_{35}O_5N_3$ Calculated.   C 64.67, H 7.92.
                        Found.   " 64.65, " 7.71.

Isosarmentogenin.—Because of the sparing solubility of sarmentogenin, it was found advisable after a number of attempts to proceed as follows for the isomerization.   0.5 gm. of the powdered genin was suspended in 2.5 cc. of anhydrous pyridine and the mixture was treated at 0° with 2.5 cc. of a solution of potassium hydroxide in dry methyl alcohol which contained 4.8 gm. per 100 cc.   After shaking at 0° solution was complete after several hours, and after 5 hours the Legal reaction had become practically negative.   The solution was diluted and treated with an excess of dilute sulfuric acid until strongly acid to Congo red.   This caused the precipitation of amorphous material.   The mixture was shaken out with chloroform after standing for 1 day to complete the relactonization of any saponified material.   The washed chloroform extract was then washed again with dilute sodium carbonate solution to remove acid products and dried.   The concentrated extract left a residue which crystallized under 95 per cent alcohol.   The yield was about 25 to 30 per cent.

When recrystallized from alcohol, the iso compound separates as stout needles and prisms which contain 1 mol of solvent and melt at 248°.

Air-Dry Substance.
4.258 mg. substance: 2.328 mg. AgI.
$C_{23}H_{34}O_5 \cdot C_2H_5OH$. Calculated. OC_2H_5 10.31. Found. OC_2H_5 10.50
Anhydrous Substance.—Dried at 100° and 30 mm.
3.654 mg substance: 2.918 mg. H_2O, 9.475 mg. CO_2.
4.093 "   "   : 3.195 "   "   10.635 "   "
                $C_{23}H_{34}O_5$.   Calculated.   C 70.72, H 8.78.
                        Found.   " 70.71, " 8.93.
                          "   " 70.85, " 8.75.

When recrystallized by dilution of its concentrated alcoholic solution with water, it separated as dendritic aggregates of lustrous, four-sided leaflets which melted at 248° and now contained water of crystallization. The air-dry substance gave a negative Zeisel test and lost 5.34 per cent of water when dried at 100° and 30 mm. Its solution in sulfuric acid develops a yellow color which slowly deepens to a deep brown-red. The iso compound does not give the Legal reaction.

Isosarmentogenin Dibenzoate.—This was prepared as in the case of the derivative of the parent compound and was recrystallized by the addition of alcohol to a concentrated solution in acetone. It separated as anhydrous, glistening, narrow prisms which melted at 297°.

2.920 mg. substance: 1.870 mg. H_2O, 7.900 mg. CO_2.
$C_{47}H_{42}O_7$.   Calculated.   C 74.21, H 7.08.
                 Found.   " 73.78, " 7.20.

Isosarmentogenic Acid.—A solution of 0.8 gm. of isosarmentogenin in 5 cc. of pyridine was treated with 16 cc. of 0.5 N NaOH. After complete solution had occurred, the mixture was left a few minutes to insure saponification of the lactone group. The solution was carefully neutralized to phenolphthalein with acetic acid and then treated with 8 cc. of a solution of bromine in N NaOH containing 70 mg. per cc. After 1 hour, acidification produced an amorphous precipitate which gradually crystallized on longer standing. When recrystallized from dilute alcohol, the lactone acid formed lustrous platelets which melted with slow effervescence at 212°. It is soluble in alcohol, but sparingly so in acetone and practically insoluble in chloroform and ether. In sulfuric acid the solution, at first colorless, gradually deepens to a yellow color.

The substance was dried for analysis at 100° and 30 mm. The hygroscopic character of the dried material, however, rendered analysis somewhat difficult.

5.214 mg. substance: 3.880 mg. H_2O, 12.945 mg. CO_2.
3.412 "   "   : 2.590 "   "   8.470 "   "
$C_{23}H_{34}O_6$.   Calculated.   C 67.99. H 8.44.
                 Found.   " 67.70, " 8.32.
                   "   " 67.70, " 8.50.

That the substance is a lactone acid was shown by titration.

9.970 mg. of the substance were treated with 1 cc. of alcohol and directly titrated with 0.1 N NaOH against phenolphthalein. Found 0.249 cc.   Calculated for 1 equivalent, 0.245 cc.

The above solution was then treated with 2.60 cc. of 0.1 N NaOH and refluxed for 3.5 hours, and then titrated back.   Found, 0.256 cc.   Calculated for 1 equivalent 0.245 cc.

Isosarmentogenic Methyl Ester.—When a suspension of the previous acid in acetone was treated with diazomethane, it was replaced without apparent solution by the very sparingly soluble ester. When recrystallized by concentration of its solution in hot acetone, it formed lustrous, four-sided platelets which melted with effervescence at 274°.   It is very sparingly soluble in the usual solvents.

4.043 mg. substance: 3.235 mg. H_2O, 10.153 mg. CO_2.
4.330 "   "   : 3.380 "   "   10.900 "   "
$C_{24}H_{34}O_4$.   Calculated.   C 68.53, H 8.64.
                    Found.   " 68.48, " 8.95.
                      "   " 68.64, " 8.74.

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